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THE EFFECT OF CALCIUM AND PHOSPHORUS ON THE METABOLISM OF LEAD

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(Received for publication December 6, 1940)

The greater part of the lead hazard to which the average American is exposed and which Minot ('38) has recently assessed, is contributed by the food, and of the intake of lead in the food, an undetermined but probably not inconsiderable fraction is contributed by fruits that have been sprayed with lead arsenate for the control of insect pests during the development of the fruit. The fact that the Federal tolerance for lead has been raised in the last 2 years from 0.019 to 0.050 grain per pound of food affords some basis for the belief that the lead intake of the average American will increase in proportion to the consumption of fruit sprayed with lead arsenate. The fact that these usual and presumably sub-toxic levels of lead consumption seem to induce a slow but significant accumulation of lead in the human body (Morris, '40), should not be lost sight of in this connection.

Since modern life inevitably involves a lead hazard, it is important to explore the possibility of minimizing its effects on the body. The intake of lead, like the intake of calcium, is not a good measure of the physiological effect to be expected, since the body assimilates dietary components to different degrees of completeness depending among other things upon the direct effect of one dietary component on the assimilation of another. As an instance of such a relationship may be mentioned the marked effect of some apple constituent or constituents in depressing the assimilation of lead (Shields, Mitchell and Ruth, '39 b).

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The element calcium seems to possess to a preëminent degree the property of disturbing the assimilation of other minerals. Its relation to phosphorus assimilation is well known. The utilization of phytic acid in particular is impaired by calcium to phosphorus ratios of 2 to 1 or higher (Krieger and Steenbock, '40). Many salts of calcium greatly reduce the assimilation of iron (Kletzien, Buchwald and Hudson, '40), while high ratios of calcium to phosphorus produce the same effect (Anderson, McDonough and Elvehjem, '40). Excess calcium in the diet of chicks depresses the assimilation of manganese (Wilgus and Patton, '39), this effect resulting apparently from interactions between the two elements in the digestive tract (Caskey and Norris, '39). In a similar manner, an excess of calcium will impair the assimilation of iodine (Thompson, '36). However, these effects of excess dietary calcium are not all detrimental to the animal, since a high level of calcium in the diet may counteract the deleterious effects of high levels of iodine (Thompson, '36) and of manganese (Becker and McCollum, '38).

The retention of lead in the body and its detrimental effects on physiological processes may also be counteracted by excess dietary calcium. In fact, the triumvirate of factors controlling calcification of the bones, dietary calcium, phosphorus and vitamin D, have been claimed to modify the retention of lead. Sobel, Yuska, Peters and Kramer ('40) have shown that the addition of either calcium or phosphorus to a low-calcium low-phosphorus diet containing added lead will depress lead retention in rats, while the addition of vitamin D to any of the types of diets studied by them will enhance lead retention. Shelling ('32–'33) and Shelling and Hopper ('36) emphasize in particular the effect of phosphorus in the control of lead toxicity. Calvery and his associates (Grant, Calvery, Laug and Morris, '38), also, found with rats that lead retention was decreased by increasing the calcium content of the diet, but at the same time the phosphorus content of the diet was lowered. The experiments of Tompsett ('39) and of Tompsett and Chalmers ('39) on adult mice confirm the depressing effect

of high-calcium diets on lead retention. That the effect of calcium on the assimilation of lead is dependent mainly or entirely upon events occurring in the digestive tract, is shown by the experiments of Lederer and Bing ('40). In these experiments, the retention of lead injected intraperitoneally into rats was independent of the calcium and phosphorus contents of the diet, while lead incorporated in the diet was retained in much greater amount in bone and kidney, but not in liver, when the calcium content of the diet was low than when it was high.

Most of the experiments on the effect of calcium, phosphorus and vitamin D on the metabolism of lead involved the use of diets containing much higher concentrations of lead than would be of significance in practical nutrition. Shelling used rations containing 1.2% of lead; Sobel used rations containing 0.80 to 0.82% of lead, with much higher concentrations in his earlier work; Tompsett in most of his experiments fed 1 mg. of lead to each mouse daily, equivalent to 0.04% of the ration; Lederer and Bing fed diets containing 100 p.p.m. of lead. Calvery and his associates ('38), in their studies of similar import, fed diets varying in lead content from 13 to 512 p.p.m.

It seemed important to re-explore the field of the relationships between dietary calcium, phosphorus and lead from the standpoint of the lead hazard existing in the American diet. Are the relationships the same for low concentrations of dietary lead as for the high concentrations usually studied in the past? The present Federal tolerance for lead is equivalent only to 7.1 p.p.m. of fresh fruit, or about 42 p.p.m. of dried fruit (apple).

PLAN OF EXPERIMENTS

The experiments were planned to compare the retentions of lead in growing and adult rats induced by diets containing a constant proportion of lead, but variable proportions of calcium with phosphorus constant, of phosphorus with calcium constant, and of both calcium and phosphorus, the variations being simultaneous. The lead concentration of the diets varied

from about 15 to 33 p.p.m. of dry matter. The lead was added in all cases in the form of acid arsenate, $PbHAsO_4$, commonly used as a spray chemical.

The composition of the basal diets used in the five experiments to be reported in this paper is given in table 1. To these basal diets, supplements of $PbHAsO_4$, $CaHPO_4$, Na_2HPO_4 and $CaSO_4$ were added to give the desired concentrations of lead, calcium and phosphorus.

TABLE 1

Description of experimental basal diets, expressed in per cent of ingredients

INGREDIENTS	BASAL DIETS USED IN EXPERIMENTS NUMBER			
	184 and 185	229	165	212
Dried and extracted beef	12.5	0	0	12.0
Dried and extracted egg	12.5	0	23.0	0
Egg albumin	0	20.0	0	0
Butter fat	4.0	0	6.0	0
Lard	8.0	0	9.0	12.0
Corn oil	0	12.0	0	0
Sucrose	10.0	10.0	10.0	10.0
Starch	32.5	41.5	24.5	48.5
Agar	1.0	1.0	1.0	1.0
Cod liver oil	1.0	1.0	1.0	1.0
Wheat germ oil	0.5	0.5	0.5	0.5
Dried yeast	10.0	6.0	10.0	10.0
Salt mixture (Ca- and P-free)[1]	8.0	8.0	15.0	5.0
Total	100.0	100.0	100.0	100.0

[1] Contains from 50 to 67% starch to facilitate mixing.

The rats were fed in groups of two, three or four, depending upon the number of rations to be compared, and within each group the food intakes were equalized. Each group of rats was of the same sex, and generally of the same litter, and were selected to be approximately equal in body weight. Representative rats from each litter contributing rats to the experiment were sacrificed and analyzed for lead, and for calcium and phosphorus when retentions of these elements during the experimental feeding periods were estimated.

This equalization of the food intakes of grouped rats on comparable rations is considered to be an essential feature

of the experiments, since only under these conditions will the intakes of lead, calcium and phosphorus among the rations to be compared be in the desired proportions, and only under these conditions will the intake of other nutrients be the same. Hence, only under conditions of equal intakes of food can the results be interpreted strictly in terms of the differences in composition of the experimental rations.

At the termination of the feeding periods, lasting generally from 7 to 22 weeks, the rats were killed, the body length from tip of nose to root of tail measured, and the empty carcass of each analyzed for lead and occasionally for calcium and phosphorus, either in the whole carcass or in bones and soft tissues, separated after autoclaving. Each group of rats was sacrificed when it had consumed a definite amount of food, the same for each group in its respective experiment. The amounts of food consumed were generally 500 gm., 600 gm., or 1000 gm. per rat.

The method of lead analysis used is a modification of the dithizone method, described in a previous publication from this laboratory (Shields, Mitchell and Ruth, '39 a). The calcium analyses were carried out according to the method of McCrudden ('11–'12) and the phosphorus analyses according to the official method of the A.O.A.C. for phosphorus in fertilizer.

DISCUSSION OF EXPERIMENT RESULTS

The average results of the lead metabolism studies are summarized in table 2; the average results on the distribution of the retained lead between bone and soft tissues, insofar as this was determined, will be found in table 3, while the limited amount of data on calcium and phosphorus retention and on the content of these elements in the experimental carcasses is assembled in table 4. Table 5 contains statistical analyses according to the method of Student ('08; '25) of the significant comparisons of the average data reported in the preceding three tables.

The results of experiments 229 and 185 (table 2) show that diets containing 0.12 and 0.38% of phosphorus, and calcium

TABLE 2

Lead metabolism data. Averages per rat

EXPT. NO.	COMPOSITION OF DIETS:			NUMBER OF RATS IN GROUP	DAYS ON TEST	TOTAL FOOD CONSUMED	INITIAL BODY WEIGHT	TOTAL GAIN IN BODY WEIGHT	ATTAINED BODY LENGTH	TOTAL LEAD IN CARCASS	ESTIMATED LEAD RETENTION	
	Calcium	Phosphorus	Lead								Total	Per cent of intake
	%	%	*p.p.m.*			*gm.*	*gm.*	*gm.*	*mm.*	*mg.*	*mg.*	
185	0.55	0.38	15.8	6	76	600	42	134	204	0.380	0.269	2.8
	0.53	0.68	15.3	6	76	600	41	136	204	0.215	0.104	1.1
	0.54	1.14	15.7	6	76	600	41	117	201	0.252	0.141	1.5
229	0.19	0.12	23.7	11	73	500	45	98	184	0.868	0.823	6.9
	0.19	0.43	23.1	11	73	500	45	97	184	0.601	0.557	4.8
	0.18	0.82	27.3	11	73	500	44	89	183	0.607	0.562	4.1
184	0.27	0.53	16.6	4	73	600	37	162	209	0.339	0.231	2.8
	0.54	0.53	16.8	4	73	600	36	148	206	0.197	0.089	0.9
	1.11	0.54	16.6	4	73	600	36	147	208	0.231	0.123	1.2
165	0.10	0.37	16.9	11	156	1001	52	148	216	2.180	2.122	12.5
	0.65	0.61	16.7	11	156	1001	51	155	216	1.019	0.961	5.7
	1.08	1.06	17.2	11	156	1001	52	154	217	0.484	0.426	2.4
212	0.03	0.27	2.1	8	48	481	395	— 9	—	0.272	0	—
	0.03	0.26	32.6	8	47	475	392	— 27	—	1.360	1.088 [1]	7.6 [2]
	0.12	0.32	32.1	8	48	481	393	— 12	—	1.174	0.903 [1]	6.2 [2]
	0.46	0.34	29.8	8	48	481	397	— 13	—	0.447	0.175 [1]	1.3 [2]

[1] Lead retentions above that of rats on basal diet containing no added lead.

[2] Based on lead intake above that of rats on basal diet containing no added lead.

to phosphorus ratios of 1.4:1 and 1.6:1, respectively, definitely promote retention of the contained lead, as compared with diets containing larger proportions of phosphorus but no more calcium. However, concentrations of phosphorus greater than 0.43% in experiment 229 and 0.68% in experiment 185, did not depress lead storage further. In fact, there was a

TABLE 3

Distribution of lead stores in body. Averages per rat

EXPT. NO.	COMPOSITION OF DIETS:			NUMBER OF RATS IN GROUP	LEAD CONTENT:		PER CENT OF TOTAL BODY Pb IN SOFT TISSUES
	Calcium	Phosphorus	Lead		In soft tissues	In bones	
	%	%	*p.p.m.*		*mg.*	*mg.*	
165	0.10	0.37	16.9	6	0.246	1.875	11.62
	0.65	0.61	16.7	6	0.156	0.875	15.11
	1.08	1.06	17.2	6	0.087	0.421	17.25
212	0.03	0.27	2.1	5	0.082	0.189	31.2
	0.03	0.26	32.6	5	0.195	1.112	15.0
	0.12	0.32	32.1	4	0.197	0.989	19.2
	0.46	0.34	29.8	5	0.149	0.382	24.8

TABLE 4

Calcium and phosphorus metabolism data. Averages per rat

EXPT. NO.	COMPOSITION OF DIETS		CALCIUM IN CARCASS		ESTIMATED CALCIUM RETENTION	PHOSPHORUS IN CARCASS		ESTIMATED PHOSPHORUS RETENTION
	Calcium	Phosphorus						
	%	%	*gm.*	%	*gm.*	*gm.*	%	*gm.*
229	0.19	0.12	1.166	0.82	0.748	0.869	0.614	0.567
	0.19	0.43	1.194	0.85	0.774	0.881	0.623	0.577
	0.18	0.82	1.140	0.86	0.724	0.838	0.628	0.537
165	0.10	0.37	1.592[1]	0.83	—	1.182	0.60	—
	0.65	0.61	2.009[1]	0.98	—	1.307	0.64	—
	1.08	1.06	2.097[1]	1.04	—	1.386	0.69	—

[1] In bones or carcass.

tendency at the higher phosphorus levels, though it was not significant statistically, for the lead retentions to be somewhat greater than at the intermediate levels.

The decrease in lead retention from the low to the medium phosphorus level in experiment 229, was not associated with an appreciable or significant increase in the retention of either

TABLE 5

Statistical analysis of results reported in tables 2, 3, and 4

EXPT. NO.	RATIONS COMPARED	DESCRIPTION OF EXPERIMENTAL DATA	STATISTICAL RESULTS: Average difference	Standard deviation	Probability [1]	NUMBER OF PAIRED DIFFERENCES
185	Low P — medium P	Pb retention, mg.	0.165	0.115	0.012	6
	Low P — high P	" " "	0.128	0.113	0.026	6
	High P — medium P	" " "	0.037	0.091	0.20	6
229	Low P — medium P	Pb retention, mg.	0.266	0.284	0.007	11
	Low P — high P	" " "	0.261	0.374	0.026	11
	High P — medium P	" " "	0.006	0.218	0.47	11
	Medium P — low P	Ca retention, gm.	0.0260	0.0478	0.058	11
	Medium P — high P	" " "	0.0501	0.0563	0.009	11
	Low P — high P	" " "	0.0241	0.0377	0.035	11
	Medium P — low P	P retention, gm.	0.0100	0.0285	0.15	11
	Medium P — high P	" " "	0.0401	0.0334	0.0017	11
184	Low Ca — medium Ca	Pb retention, mg.	0.142	0.069	0.021	4
	Low Ca — high Ca	" " "	0.108	0.091	0.067	4
	High Ca — medium Ca	" " "	0.034	0.057	0.10	4
165	Low (Ca + P) — medium (Ca + P)	Pb retention, mg.	1.161	0.253	<0.00001	11
	Low (Ca + P) — high (Ca + P)	" " "	1.696	0.270	<0.00001	11
	Medium (Ca + P) — high (Ca + P)	" " "	0.535	0.145	<0.00001	11
	Low (Ca + P) — medium (Ca + P)	Pb in soft tissues, mg.	0.090	0.041	0.0023	6
	Medium (Ca + P) — high (Ca + P)	" " " " "	0.069	0.033	0.0027	6
	Medium (Ca + P) — low (Ca + P)	P in carcass, gm.	0.125	0.080	0.0003	11
	High (Ca + P) — medium (Ca + P)	" " " "	0.079	0.099	0.014	11
	Medium (Ca + P) — low (Ca + P)	Ca in carcass, gm.	0.649	0.446	0.022	5
	High (Ca + P) — medium (Ca + P)	" " " "	0.089	0.328	0.31	5
	Medium (Ca + P) — low (Ca + P)	Ca in bones, gm.	0.223	0.135	0.0072	6
	High (Ca + P) — medium (Ca + P)	" " " "	0.088	0.073	0.021	6
212	Low Ca — medium Ca	Pb retention, mg.	0.185	0.344	0.10	8
	Medium Ca — high Ca	" " "	0.728	0.395	0.0009	8
	High (Ca + Pb) — low Ca (no added Pb)	" " "	0.175	0.175	0.017	8
	Low Ca — high Ca	Pb in soft tissues, mg.	0.046	0.030	0.019	5
	Medium Ca — high Ca	" " " " "	0.057	0.051	0.074	4

[1] Probability to fortuitous factors alone would bring about an average difference as great as, or greater than, the observed average difference.

calcium or phosphorus (table 4). The retention of both calcium and phosphorus was definitely depressed when the phosphorus level was raised to 0.82%, with a calcium to phosphorus ratio of 0.22:1. Evidently in this experiment there was no tendency for lead deposition to parallel calcium or phosphorus deposition. On the contrary, the relationship tended to be an inverse one.

Experiment 184 permits a study on growing rats of the effect on lead deposition of increasing levels of calcium, the phosphorus level remaining constant. As the calcium content of the diet increased from 0.27 to 0.54%, the retention of lead decreased to less than half. A further increase in the calcium level to 1.11 and in the Ca: P ratio from 1:1 to approximately 2:1, produced an inappreciable and statistically insignificant increase in the deposition of lead.

Experiment 212 permits the same sort of a comparison with mature rats. Here an increase in the level of calcium in the diet from 0.03 or 0.12 to 0.46% produced a marked and significant reduction in lead storage, both in the entire carcass, in the bones and in the soft tissues (table 3). The reduction in the lead content of the soft tissues by an increase in the calcium content of the diet to only a moderate level, seems especially significant. With the same lead content in a diet containing 1.24% of calcium, a previous experiment (Shields, Mitchell and Ruth, '39 a) had failed to demonstrate any retention of lead in mature rats.

From table 3 it may also be seen that the proportion of the total content of lead in the carcass that is present in the soft tissues varies inversely as the total content. The results of experiment 165 also support this statement. For low contents of lead, 25 to 30% may be present in the soft tissues.

In experiment 165, the effect on the retention of lead in young rats of increasing simultaneously the proportion of dietary calcium and phosphorus was tested. Here the lead retention and the lead content of the carcass, bones and soft tissues decreased greatly and significantly with the increasing mineral content of the diet. On the contrary the calcium and phosphorus contents of the carcass and the calcium content of

the bones increased with the increase in the concentration of these elements in the diet, especially in the comparison of the low-mineral and medium-mineral diet. Here again the retention of calcium responds to a change in the calcium content of the diet in a way diametrically opposed to that of the retention of lead.

The results of these experiments do not appear to conform with the theory of Sobel, that the factors controlling the deposition of lead in the bones are similar to those controlling the assimilation of calcium and in particular that there is a most favorable ratio of lead to phosphorus of approximately 3 to 1. Nor do they conform with the theory of Shelling, which emphasizes the importance in lead assimilation of the presence of available phosphorus in the diet. Furthermore, they demonstrate that the lead stream from the intestinal tract to the tissues may not vary with the calcium stream.

There would seem to be at least two spheres of reaction within which the effects of dietary calcium and dietary phosphorus on lead assimilation are mediated. One sphere is the lumen of the gastrointestinal tract, where the absorption of dietary lead appears to be impaired by concentrations of calcium or phosphorus or both above certain low limits. The other sphere is the area of mineral interchange between blood and bone trabeculae. The less active this interchange becomes, due either to advancing age or to the saturation of mineral stores, the less opportunity is there for an active deposition of any bone mineral, including lead.

SUMMARY AND CONCLUSIONS

The effect of varying the concentration of calcium and phosphorus in diets containing low concentrations of lead (16 to 32 p.p.m.) on the retention of lead was investigated in a series of five experiments involving 128 growing or adult rats. In some of these experiments the retention of calcium and phosphorus was also measured. All mineral retentions were measured by carcass analysis. An essential feature of the experiments was the equalization of the food intakes of rats on comparable diets, thus permitting the interpretation

of the results in terms solely of the relative compositions of the diets.

The results obtained appear to warrant the following conclusions:

1. A low content of calcium or of phosphorus or of both in the diet induces a high retention of lead in comparison with diets containing higher mineral levels. In fact, the only method of securing lead storage in adult rats on the moderate concentration of dietary lead used (32 p.p.m.) is to lower the calcium content of the diet to inadequate or borderline levels.

2. Excessive dietary levels of calcium and phosphorus are not appreciably more protective against the assimilation of lead by the body than are levels approximating the requirements. This statement applies only to the moderate levels of dietary lead employed.

3. Under the imposed conditions of variable dietary concentrations of calcium and phosphorus, the retention of calcium runs in a diametrically opposite direction to the retention of lead.

4. Under conditions of practical nutrition, an adequate intake of calcium and of phosphorus presumably protects the body against appreciable assimilation of the low levels of dietary lead involved in the usual lead hazard of modern life. This protection is more effective in the adult than in the adolescent for any given concentration of calcium and phosphorus, possibly because the mineral metabolism of the bone trabeculae is considerably less intense in the adult than in the growing organisms.

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RESPIRATORY METABOLISM IN FRUCTOSURIA [1]

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ONE FIGURE

INTRODUCTION

Fructosuria is a disturbance of metabolism in which part of the fructose ingested is excreted in the urine. It may occur uncomplicated by other disturbances of carbohydrate metabolism, or it may appear in combination with the excretion of glucose. This paper will deal with the former condition, essential fructosuria.

The first case of probable fructosuria presented in the literature is that of Zimmer and Czapek in 1876. Including this case, Silver and Reiner, reporting on three additional patients, list a total of twenty-seven cases of fructosuria up to 1934. Recent observations besides those of Silver and Reiner ('34) have been reported by Heeres and Vos ('29), Marble and Smith ('36), van Lookeren Campagne ('36), and Edhem, Erden and Steinitz ('38). These investigators describe the disorder as characterized by the excretion of a constant proportion of ingested fructose, amounting to between 10 and 20%, and an elevation of the total blood sugar level due to the presence of fructose despite a small depression in blood glucose.

In 1935 Blatherwick (unpublished data) noted the absence of any appreciable rise in blood lactate following fructose ingestion in two individuals with fructosuria (subject A. O.

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of this paper and his sister). Recently Edhem, Erden and Steinitz ('38) found similar results with respect to blood lactic acid in their subject. The only respiratory metabolism data are those of Heeres and Vos who report that their subject showed a rise in quotient to 0.77 from a basal level of 0.72 one hour after fructose ingestion, compared with a rise from the basal quotient of 0.71 to a quotient of 0.95 one hour after fructose ingestion in a normal control. Although data beyond the first hour after fructose ingestion are not given, the authors conclude that no combustion of sugar takes place. Therefore a more complete study of the respiratory metabolism and its relation to the lactic acid changes in the blood was undertaken.

EXPERIMENTAL DATA

Of the four available subjects, only two were sufficiently cooperative for reliable respiratory metabolism data. We are indebted to Drs. B. S. Oppenheimer and S. Silver for referring subject A. R., who is case 1 in the report of Silver and Reiner. He is of Italian parentage, male, 29 years old, 157 cm. tall, and weighs 53 kg. Subject A. O. is a deaf-mute of Hungarian extraction, male, age 25 years, height 167 cm., and weighs 77 kg. A younger brother of A. R., as well as the sister of A. O., also show the disorder.

Respiratory metabolism was determined in approximately 30-minute periods with the Benedict Universal apparatus. The data presented in table 1 have been calculated on the basis of 30-minute periods, disregarding the necessary 6- to 10-minute intervals between periods. The subjects came to the laboratory in the post-absorptive state. After resting for about an hour the basal metabolism level was established and, except where otherwise noted in table 1, 50 gm. of sugar in 250 cc. of water at 37°C. were given orally. The metabolism determinations were continued until the subject returned to approximately the basal level.

Urines were collected for the basal periods and after each sugar experiment, according to the time intervals shown in

TABLE 1

Changes in non-protein respiratory quotients and heat production following ingestion of sugar

DATE	INGESTED		30-MINUTE PERIODS	BASAL METABOLISM					AFTER INGESTION OF SUGAR								
	Sugar	H_2O		1	2	3	4	Av.	1	2	3	4	5	6	7	8	9
Subject A. R.																	
	gm.	*cc.*															
7/17	50	250	R.Q.	0.78	0.78	0.75	0.74	0.76	0.72	0.83	0.83	0.90	0.87				
	gluc.		Cals.	28.0	27.5	28.2	28.7	28.1	30.2	30.3	28.5	31.4	27.9				
7/24	50	250	R.Q.	0.75	0.79	0.74		0.76	0.82	0.87	0.87	0.91	0.82	0.72	0.72		
	fruct.		Cals.	27.7	27.6	28.6		28.0	27.6	28.7	30.0	29.2	30.7	28.3	28.5		
7/31 [1]	50	500	R.Q.	0.73	0.71	0.71	0.70	0.71	0.73	0.73	0.81	0.83	0.78	0.74	0.73	0.72	
	fruct.		Cals.	27.7	27.7	28.0	27.7	27.8	29.5	30.2	30.6	31.1	30.1	29.8	28.9	29.3	
Subject A. O.																	
3/13	50	250	R.Q.	0.80	0.84	0.90	0.84	0.85	1.04	0.82	0.85	0.83	0.84				
	fruct.		Cals.	36.7	35.6	34.5	33.9	35.2		37.2	35.6	36.2	35.9				
3/20	50	250	R.Q.	0.88	0.84	0.77		0.83	0.81	0.82	0.84	0.83	0.85				
	fruct.		Cals.	34.3	34.9	35.2		34.8	34.6	36.5	36.2	37.0	36.6				
4/3	50 + 50	250 + 250	R.Q.	0.88	0.83	0.84		0.85	0.89	0.86	0.87	0.87	0.84 ↑ ↓ 35.5	0.93 36.4	0.93 36.4	0.88 36.5	0.88 36.4
	fruct.		Cals.	34.4	35.5	35.9		35.2	35.3	35.7	36.9	37.1					
4/10	50 + 50	250 + 250	R.Q.	0.92	0.90	0.86	0.89	0.89	0.88	0.85	0.91	0.89 ↑ ↓ 36.3	0.95 35.8	0.91 36.4	0.83 37.0		
	fruct.		Cals.	32.7	33.4	33.5	34.4	33.5	34.8	35.1	36.3						
6/26 [2]	50 + 50	250 + 250	R.Q.	0.88	0.86	0.80		0.84	0.92	0.89	0.91	0.91 ↑ ↓ 36.6	0.92 37.8	0.90 37.9	0.81 36.6	0.83 39.1	
	fruct.		Cals.	35.2	36.2	36.0		35.8	35.8	36.6	37.0						

↕ Second dose of fructose.

[1] Subject on a low carbohydrate intake for 2 days previous to this experiment.

[2] Bloods taken for fructose determination during this experiment.

table 3. Nitrogen was determined by the Kjeldahl method and fructose according to Roe ('34). The latter agreed within 0.3% with the total sugar by the Shaffer-Somogyi method ('33), and by polariscope, after clarifying the urine with neutral lead acetate.

TABLE 2

Lactic acid and sugar concentration in blood following fructose ingestion

SUBJECT	DATE	VENOUS BLOOD	HOURS AFTER FRUCTOSE INGESTION						
			0	0.5	1	2	3	4	6
			mg.%	*mg.%*	*mg.%*	*mg.%*	*mg.%*	*mg.%*	*mg.%*
A.R.	8/7/37	Lactic acid	9.0	7.1	10.8	11.4		12.2	
		Fructose	0	40	48	54		12	
		Glucose	93	87	91	86		84	
		Total sugar	93	125	137	138		95	
A.R.	1/8/38	Lactic acid	9.0	7.7	7.0	8.2		10.1	10.1
		Fructose	0	29	45	42		17	Trace
		Glucose	82	73	70	68		73	80
		Total sugar	82	101	113	109		89	80
A.O.	4/17/35	Lactic acid	10.8	13.8		10.9	10.1		
		Fructose	0	40		43	22		
		Glucose	86	76		81	83		
		Total sugar	86	116		124	105		
A.O.	6/26/37	Lactic acid	7.1	7.2			13.5	13.3[1]	
		Fructose	Trace	23			36	47	
		Glucose	78	76			74	74	
		Total sugar	78	98			108	119	

[1] Forty minutes after second dose of fructose.

The blood fructose and lactate experiments were conducted on different days from the respiratory metabolism experiments except the experiment on A. O. of June 26th. The same precautions as to basal conditions and administration of sugar were observed. Proteins were precipitated from 5-cc. samples of venous blood by the Somogyi ('30) procedure, and the total blood sugar determined according to Shaffer and Somogyi ('33), the fructose by Roe's method, and the lactate by the method of Friedemann and Graeser ('33) as modified by Blatherwick et al. ('35). Chemically pure sugars were administered. This is essential in the case of fructose to prevent the diarrhoea that is occasioned by its impurities (Rowe, Plummer and McManus, '33).

Control experiments were carried out on three normal subjects. Since the results agree with those established by other investigators (Deuel, '36), the data are not included in this paper.

The metabolism studies on the two fructosuric subjects are shown in table 1. The first observations on subject A. R. (July 17th) were a control test with 50 gm. of glucose. The reaction was similar to that in the normal individual, a slow rise in non-protein respiratory quotient which reached a peak at 0.90 in the fourth period, about 2½ hours after ingestion of the sugar (Cathcart and Markowitz, '27; Carpenter and Fox, '30 a). The respiratory quotients after 50 gm. of fructose (July 24th) followed the same curve to a high point of 0.91 in the fourth period, thus indicating an oxidation of fructose similar to that of glucose in this individual. In order to reduce the carbohydrate metabolism a diet low in carbohydrate, limited to 50 gm. of lactose in milk, was taken for 2 days prior to the fructose experiment of July 31st. The basal R.Q. was low, 0.71, and ketones were present in both basal and post-fructose urines. The rise in respiratory quotient after fructose ingestion followed the same curve as previously, but at a lower level, with the highest quotient at 0.83, thus agreeing with glucose studies on normal individuals under similar dietary conditions (Chambers, '38). These results suggest a transformation of fructose into glucose with subsequent oxidation of the latter sugar.

The basal metabolism of subject A. R. remained at the same level during the three experiments (table 1), averaging 37.3 calories per square meter per hour or —8 according to the DuBois standard. Following the ingestion of fructose the rise in heat production was similar to that after glucose was taken, reaching in the fourth or fifth period a maximum specific dynamic action of about 6 calories per hour, which is within the limits found in the normal individual (Carpenter and Fox, '30 b).

The results with the other subject (A. O.) differ from those just cited. The non-protein respiratory quotients for the

basal periods were consistently higher, averaging 0.85, although the individual periods show a greater variation. Following the ingestion of 50 gm. of fructose, no change in quotient occurred which could be considered significant in four of the five experiments. The single high quotient of 1.04 for the first period after ingestion of fructose in the first experiment (March 13th) can be discarded on the basis of a high respiratory rate and probable hyperventilation. Therefore the heat production for this period has not been calculated. Thus it appears that little if any stimulus to carbohydrate oxidation occurred in this subject from the ingestion of 50 gm. of fructose. Since Wierzuchowski ('26) obtained a rise in respiratory quotient from phlorhizinized animals after a second dose of glucose which was not demonstrable after the first dose, and similar results were found in normal animals after fasting (Dann and Chambers, '30), a second 50 gm. portion of fructose was taken by subject A. O. 2 to 3 hours after the first dose as noted in table 1. This led to a distinct rise in the quotient to 0.93 on April 3rd and 0.95 on April 10th.

The last experiment (June 26th) disagreed with the other four in that the quotient was maintained at a constant high level of about 0.91 throughout the four periods after the first dose of fructose and for the first two periods after the second dose of sugar. It then returned to the basal level. The technique of this experiment was different from the two preceding ones in that during the experiment blood samples were drawn at the intervals shown in table 3.

Despite the high level of carbohydrate metabolism in the basal periods of this fructosuric subject, 50 gm. of fructose were insufficient to stimulate a significant increase in sugar oxidation, although a second 50-gm. dose was effective.

The basal metabolism was 37.8 calories per square meter per hour or a B. M. R. of —4. The maximum extra heat production following the ingestion of fructose averaged about 4.5 calories per hour in the absence of a definite rise in respiratory quotient, a reaction similar to that found in the impaired

TABLE 3

Urinary excretion of nitrogen and fructose after ingestion of fructose

SUBJECT	DATE	BASAL N/HR.	FIRST 50 GM. FRUCTOSE				SECOND 50 GM. FRUCTOSE			
			Period	N/hr.	Total fructose	Per cent of ingested fructose	Period	N/hr.	Total fructose	Per cent of ingested fructose
		mg.	*hours*	*mg.*	*gm.*		*hours*	*mg.*	*gm.*	
A.R.	7/17/37	486	[1]	414	[1]	[1]				
A.R.	7/24/37	546	5	384	8.1	16.1				
A.R.	7/31/37	624	5	483	8.2	16.3				
A.R.	8/ 7/37	[2]	4	[2]	7.4	14.8				
A.R.	1/ 8/38	[2]	6	[2]	8.8	17.6				
A.O.	3/13/37	298	3.25	246	5.1	10.2				
A.O.	3/20/37	528	3.50	348	6.9	13.8				
A.O.	4/ 3/37	541	3.5	305	6.8	13.6	2.67	394	7.3	14.6
A.O.	4/10/37	731	2.67	329	5.2	10.4	2.5	300	7.7	15.4
A.O.	6/26/37	403	3.5	408	6.6	13.2	2.67	437	8.3	16.6

[1] Glucose given.

[2] Nitrogen excretion not determined.

glucose metabolism of the fasted normal individual (Chambers, '38).

The lactic acid concentration in the venous blood following the ingestion of fructose by these two subjects (table 2) showed changes of little significance, thus confirming the earlier observations of Blatherwick and of Edhem, Erden and Steinitz. The apparent rise at 3 hours in the second experiment with A. O. (June 26, 1937) was not increased by the administration of more fructose.

The data on blood sugar changes also are shown in table 2. The rise in blood fructose after ingestion was in the range of 40 to 50 mg. per cent and accounts for the observed rise in total sugar, since the blood glucose exhibited little change or a slight fall. These results are in accord with those of the earlier investigators.

The urinary excretion of fructose by the two subjects as shown in table 3 averaged between 1.5 and 2.0 gm. per hour or a total excretion in 3 to 5 hours of from 10 to 20% of that ingested. The figures for nitrogen excretion, used in the calculation of the respiratory metabolism, are included in table 3.

There was no excretion of glucose by subject A. R. following its ingestion in the experiment carried out on July 17th. Fructose in the basal urines was negligible.

DISCUSSION

The constancy of fructose excretion in the urine by the fructosuric subject indicates that approximately 80 to 90% of ingested fructose may follow the major pathways of normal carbohydrate metabolism, namely, oxidation and glycogen formation. The similarity in the rise in respiratory quotient after fructose and after glucose in subject A. R. (fig. 1) suggests that much of the fructose ingested by this subject may be metabolized as glucose. In the other subject a stimulating dose of fructose was usually needed before the rise in R.Q. appeared.

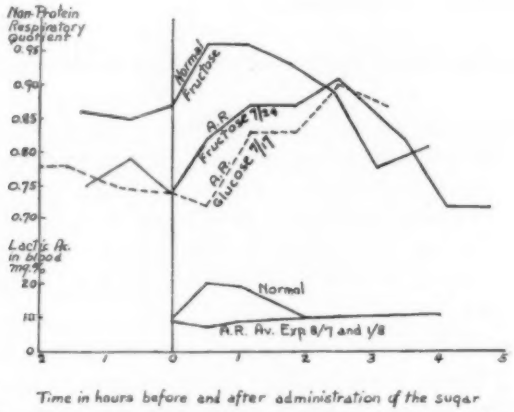

Fig. 1 The effect of ingested fructose and glucose on the respiratory metabolism and blood lactate concentration of normal and fructosuric subjects.

In the metabolism of fructose the normal individual experiences a rapid, marked rise in respiratory quotient approaching or surpassing unity and a marked increase in blood lactate, as illustrated in figure 1 by data from one of the normal control subjects. Blood sugar is only slightly elevated and none of the fructose is excreted in the urine. The early,

marked rise in both the R.Q. and in blood lactate was not found in the fructosuric subjects. A relationship between the elevated blood lactate and the high quotient is suggested by their presence in normal individuals and their absence in the fructosuric subjects.

The rapid rise in respiratory quotient following fructose ingestion by the normal individual has been ascribed either to fat formation (Cathcart and Markowitz, '27), to lactate displacement of blood CO_2 (Campbell and Maltby, '28) or to more rapid carbohydrate oxidation (Carpenter and Lee, '33; Deuel, '36; Carpenter, Bensley, Dill and Edwards, '37). Bachmann and Haldi ('37) found that the correction was a small factor in the total rise in R.Q. when the observed quotients were recalculated for excess CO_2 on the basis of displacement by lactic acid in the blood only (7% of the body weight). In the tissues of rats a rise in lactic acid after fructose ingestion has been noted by Blatherwick et al. ('40). Evidence of a rapid diffusion of lactic acid through the tissues indicates that in calculating the displacement of CO_2 the content of the intracellular tissue fluid in addition to that of the blood should be used, that is, a distribution of lactate through approximately 50% of the body weight.

Applying this correction to the average normal control experiment shown in figure 1, the obtained quotient of 0.97 becomes 0.87 on the basis of a rise in blood lactate amounting to 10 mg./100 cc. during the first ½ hour after fructose. By a similar calculation of the experiment on fructosuric subject A.R. in figure 1, if there had been a blood lactate increase of 100% after fructose ingestion such as occurred in the normal, the first quotient would have been 0.96 instead of the observed value of 0.82. These recalculated quotients from the fructosuric and normal subjects suggest that the rapid rise in respiratory quotient during the first ½ hour after fructose ingestion by the normal subject is related to the increase in lactic acid.

Since the blood lactate concentration returned to the initial level within 2 hours, a compensatory retention of CO_2 should

have occurred in this time. The rising quotient of increased carbohydrate oxidation evidently masked the compensatory fall. Thus over a period of 2 hours the acid-base changes should reach a balance and the respiratory quotient for the whole period should present a true metabolic quotient, comparable to that of the fructosuric subject who exhibits no changes in lactate.

The extent of the impaired oxidation of carbohydrate in fructosuric subject A. R. is seen in a consideration of the 2-hour period after fructose administration. During this time A. R. had an average non-protein respiratory quotient of 0.85 (table 1, 7/24). From the oxygen consumption it is calculated that he oxidized 6.32 gm. of carbohydrate per hour. For the same period the normal subject (fig. 1) had an average quotient of 0.95. On the basis of a normal quotient of 0.95, A. R. would have oxidized 10.05 gm. of carbohydrate per hour, a difference of 3.73 gm. per hour. This difference is adequate to account for the fructose excreted (table 3). The defect in carbohydrate oxidation in subject A. O. (table 1) was more striking, since a stimulating dose of fructose was required in most of the experiments before any increase in carbohydrate oxidation could be demonstrated. Although some investigators have suggested conversion of fructose to fat, the rise in quotient in the data presented here is calculated as carbohydrate oxidation, since no direct evidence for fat formation under these conditions is available.

These data emphasize two abnormal reactions to ingested fructose in the fructosuric subject, namely, a smaller than normal increase in carbohydrate oxidation and the absence of a significant rise in blood lactate. It is probably the former defect which leads to the accumulation of fructose in the blood and the consequent fructosuria, since for the normal lactate changes calculated above only about 2.5 gm. of sugar are needed. No direct relationship between the two reactions is evident. Lactic acid might be an intermediary in the transformation of fructose to glucose or a step in the direct oxidation pathway.

The authors are indebted to Mary E. Ewing and Susan D. Sawyer for the determinations of lactic acid, total sugar and fructose.

SUMMARY

The effect of ingested fructose on the respiratory metabolism, the concentration of sugar and lactic acid in the blood, and fructose excretion have been determined in two fructosuric subjects.

The rapid initial rise in respiratory quotient, characteristic in the normal individual after fructose ingestion, was absent in both subjects. One subject responded with a slow rise in R.Q. similar to that after glucose. The other usually exhibited an increase in quotient only after a stimulating dose of fructose had been taken.

Blood and urine studies also were made of two other subjects. All failed to show the normal rise in blood lactate and excreted between 10 and 20% of the ingested fructose, in agreement with the reports of earlier investigators.

The two defects in fructose metabolism, diminished oxidation and absence of a significant rise in blood lactate, are discussed. It is suggested that the former may account for the observed fructosuria.

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THE CALCIUM REQUIREMENT OF MAN: BALANCE STUDIES ON SEVEN ADULTS[1]

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In the interests of good nutrition, the adult must ingest a certain amount of calcium every day, even though the formation of new bone tissue has long since ceased—even though his skeletal tissues may already be well calcified. This need is the result of a constant daily drain of calcium from the body by way of the kidneys and intestines; if insufficient quantities of calcium are ingested habitually, this daily loss will eventually result in a depleted skeleton. Just how much calcium is necessary to prevent the occurrence of such a deficit has been critically evaluated by Sherman ('20), Leitch ('37) and Mitchell and Curzon ('39). Although these investigators made use of the same type of material, i.e., previously reported calcium metabolism data on adults, they employed different methods for their evaluations; hence, it is not surprising that they reached widely differing conclusions concerning the adult's minimal requirement for calcium. Mitchell and Curzon, in contrast to the two other investigators, pointed out a relation between the need for calcium and the size of the body and concluded that, as an average, an intake of 9.75 mg. per kilogram body weight by the adult would be sufficient to insure calcium equilibrium. Moreover, they believed that such a requirement was consistent with an average rate of utilization of dietary calcium of 30%. If Mitchell and Curzon's

[1] A portion of these data was reported before the Division of Biological Chemistry of the American Chemical Society at its annual meeting in Detroit, September, 1940.

figure for utilization is physiologic for man, then Sherman's failure to realize this may be the explanation for his low figure for requirement; i.e., 450 mg. for a 70 kg. person. However, until 1939, when Kinsman et al. and Steggerda and Mitchell published their studies on calcium utilization, there was no experimental proof of man's limited ability to make use of calcium. Furthermore, at the time the present study was undertaken only one adult, the man studied by Steggerda and Mitchell, had actually been subjected to an experiment which was specifically designed to reveal both the utilization of and the requirement for calcium; this man utilized 20% of the calcium of milk and had a total requirement of 9.2 mg. per kilogram. Obviously, more experimental data of this nature are necessary before a universally acceptable figure for calcium requirement can be obtained; for this reason the study reported here was undertaken.

EXPERIMENTAL

This study makes use of data presented in the earlier paper dealing with the calcium balances of seven healthy adults and their ability to utilize milk calcium (Breiter, Mills, Dwight, McKey, Armstrong and Outhouse, '41), and reference should be made to that work for detailed information concerning the experiment. The subjects were four women and three men—ranging in age from 21 to 42 years; they were allowed to carry on their usual activities and, at all times, reacted satisfactorily, psychologically as well as physiologically, to the conditions of the experiment. The basal dietary, which was presumably adequate in all nutrients except calcium, contributed approximately 270 mg. calcium daily. To this dietary, milk was added in quantities almost sufficient to induce calcium equilibrium, the daily amounts judged to be necessary being 180 gm. for subject Jd, 220 gm. for Jo, 260 gm. for Hb, Ws and Mh, 260 gm. (as an average) for Bm and 500 gm. for Rd. Thus, the proportion of milk calcium to non-milk calcium was practically uniform for six of the seven subjects; actually the ratios were 1.6, 1.7, 1.6, 1.4, 1.6, 1.3 and 2.7 to 1 for

subjects Hb, Bm, Jo, Ws, Mh, Jd and Rd, respectively. The milk-supplemented dietary was fed for 19 to 34 consecutive days.

RESULTS

The data which are pertinent to the assessment of the calcium requirement of these seven subjects are given in table 1; they include the quantity of calcium eaten, the resulting calcium balances and the rate at which each subject utilized the calcium of milk. On the respective daily calcium intakes of 574, 568, 498, 603, 580, 451 and 873 mg., subjects Hb, Bm, Jo, Ws, Mh, Jd and Rd had calcium balances of +24, +5, —39,

TABLE 1

The calcium requirement of seven adults

Subjects	Hb	Bm	Jo	Ws	Mh	Jd	Rd	Ave.
Weight in kg.	61	55	64	65	60	53	74	
Height in cm.	161	167	161	171	166	166	181	
Surface area in sq. m.[1]	1.64	1.61	1.66	1.75	1.64	1.57	1.93	
Total calcium intake (ave. daily) in mg.	574	568	498	603	580	451	873	
Calcium balance (ave. daily) in mg.	+24	+5	—39	—5	—13	—36	—22	
Utilization of milk calcium, %	35.1	30.6	30.3	20.1	17.6	15.3	20.4	24.2
Calcium requirement (total daily) in mg.	506	552	627	628	654	686	981	662
Calcium requirement (per kg.) in mg.	8.3	10.0	9.8	9.7	10.9	12.9	13.3	10.7
Calcium requirement (per cm.) in mg.	3.1	3.3	3.9	3.7	4.0	4.1	5.4	3.9
Calcium requirement (per sq.m.) in mg.	309	344	376	359	400	438	508	391
Milk requirement[2] (total daily) in gm.	236	281	360	313	353	405	615	

[1] Surface area was determined by the method of Boothby and Sandiford ('21).

[2] These are the quantities of milk which the subjects, in order to be in calcium equilibrium, would have to add to the non-milk foods used in this experiment.

—5, —13, —36 and —22 mg. (The wide deviation from zero shown in the balances indicates the impossibility of predicting the exact calcium requirement of a given individual.) The utilization of the calcium of milk by these seven subjects amounted to 35.1, 30.6, 30.3, 20.1, 17.6, 15.3 and 20.4%, respectively. These latter figures have been used in calculating the extent to which each subject's calcium intake would have had to be increased or decreased in order to bring about exact calcium equilibrium. Such a procedure seems reasonable particularly since none of the calcium balances departed radically from zero.

The daily calcium requirements of the subjects were computed by applying to the above data the following formula,

$$\text{Calcium Requirement} = \text{Calcium Intake} \pm \left(\frac{\text{Calcium balance}}{\%\ \text{Utilization of milk calcium}} \times 100 \right),$$

the plus sign being used for the subjects who were in negative balance, the minus sign for those in positive balance. The resulting values of 506, 552, 627, 628, 654, 686 and 981 mg. were obtained for subjects Hb, Bm, Jo, Ws, Mh, Jd and Rd, respectively. The average requirement for the group was 662 mg. of calcium.

DISCUSSION

The values reported here refer to the minimal quantity of dietary calcium needed by these seven adults for the maintenance of calcium equilibrium. Their range is great, i.e., from 506 to 980 mg. In an attempt to determine the cause of this variability, the data were evaluated in a number of ways. When converted to the conventional weight basis they became 8.3, 10.0, 9.8, 9.7, 10.9, 12.9 and 13.3 mg. per kilogram for subjects Hb, Bm, Jo, Ws, Mh, Jd and Rd, respectively, and averaged 10.7 mg. Obviously, if weight were the only determinant of calcium requirement, one would expect to find strictly similar values for all subjects instead of the wide range given here. It may be significant that both Jd and Rd, who represented the extremes in weight (i.e., 53 and 74 kg., respectively), had the same per-kilogram requirement

of approximately 13 mg.; on the other hand, subjects Bm, Jo and Ws also had virtually identical, but considerably lower, requirements (10.0, 9.8 and 9.7 mg. per kilogram, respectively). Less variability might have been found if all subjects had been equally well-padded with fat; thus the high requirement of Jd and Rd might be explained on the premise that their skeletal tissues represented a larger proportion of their weight than was true for subjects Hb, Jo and Ws who had a lower per-kilogram requirement, were it not for the fact that subjects Bm and Mh were "lean" individuals. In an attempt to rule out this factor, the total requirements were expressed on the basis of recumbent length, with resulting values of 3.1, 3.3, 3.9, 3.7, 4.0, 4.1 and 5.4 mg. per centimeter for the subjects in the usual order. On this basis, Jd's requirement of 4.1 mg. becomes more like those of Mh, Jo and Ws, who needed 4.0, 3.9 and 3.7 mg., respectively, whereas Rd again shows an extremely high requirement. This approach, like the former, yields considerable variability. The same statement can be made when the data are expressed on the basis of surface area —the values ranging from 309 to 508 mg. per square meter. To what extent the variability in requirement may have been determined by the subjects' ability to utilize calcium is not entirely clear. Although Bm and Hb, two of the best calcium utilizers, had the lowest requirements, Jo, who utilized calcium equally as well as these two subjects, had a requirement similar to those of Ws, Mh and Jd—none of whom could make use of more than 20% of his dietary calcium. Obviously, the ability of these persons to utilize calcium was not the sole determinant of their requirement—neither was their height, their weight, nor their surface area.

The average requirement for calcium (i.e., 662 mg.) computed for these seven adults is higher than those heretofore published. The 10.7 mg. per kilogram average more nearly approaches the 9.75 mg. value obtained by Mitchell and Curzon ('39) than it does the figures of Sherman ('20) or of Leitch ('37); it differs from Mitchell and Curzon's primarily in that their calculations would assign to subjects Jd and Rd, re-

spectively, 151 and 258 mg. less calcium than they actually required. Although the standard of 550 mg. recommended by Leitch (for either men or women, regardless of size) would meet the needs of subjects Bm and Hb, its application would result in serious calcium shortage for the other five—a shortage amounting to 430 mg. daily for subject Rd. On comparing these figures with that of Sherman, it is apparent that every one of the subjects had a requirement in excess of 450 mg. and that the average for the group is almost 50% greater. Sherman's figure is predicated on the basis of a body weight of 70 kg., and, when the requirements of the seven subjects are calculated according to this weight, the average becomes 752 mg., which is 67% greater than Sherman's 450 mg. figure and 10% greater than his recommended dietary standard of 680 mg.—obtained by increasing the 450 mg. value by 50% "as a margin of safety."

The reason for this disparity in calcium requirement, as determined by these two methods, warrants elucidation—especially since the same type of data (i.e., calcium balances) is used in both studies. Sherman's method (Sherman, Mettler and Sinclair, '10) was based on the sound reasoning that, inasmuch as an adult needs to eat only enough calcium to equal that which is excreted by the kidneys and intestines, his requirement can be determined by determining his total calcium excretion and that ". . . . only those experiments in which there was a reasonably close approach to equilibrium of lime . . . . can be taken as indicating the lime . . . . requirement." However, very few of the many adults studied under experimental conditions have been virtually in calcium equilibrium; Sherman himself made use of balance data which deviated radically from zero. In believing that the total calcium excretion in such cases is synonymous with requirement, he really was implying that adults could utilize 100% of their dietary calcium; e.g., if an individual is in negative balance to the extent of 150 mg., he would need to add only 150 mg. to his intake in order to be in calcium equilibrium. In

1920, this assumption was not unreasonable; today it must be appreciated that such utilization is probably never realized by man—from 15 to 35% was the range for the seven subjects of this study, and 30% was the average rate of utilization found by Mitchell and Curzon. Such an assumption does not complicate the present study—nor does it enter into Mitchell and Curzon's evaluation of the adult's requirement for calcium.

The extent to which this factor of calcium utilization will modify figures for requirement can be seen by reevaluating the data presented in the two studies. By the Sherman method, the requirement for subjects Hb, Bm, Jo, Ws, Mh, Jd and Rd, respectively, would be 550, 562, 537, 609, 593, 487 and 895 mg. and would average 605 mg. These values do not differ greatly from those computed on the basis of each one's individual capacity to utilize calcium—the reason being that the calcium balances of these subjects were not far from zero. But many of Sherman's subjects were losing more than 100 mg. of calcium daily, and, hence, their actual requirements must have been in excess of those which he assigned to them. If one assumes—in the absence of specific data—for each of his subjects a utilization rate of 30% (i.e., Mitchell and Curzon's figure derived from data on 107 adults, including these eighteen), one obtains the results recorded in table 2. In preparing this table, the balances for all periods for a given subject were averaged—hence the eighteen sets of data, rather than Sherman's original ninety-seven. For those subjects (nos. 3 through 75) who reacted to the low calcium intakes in a manner similar to that exhibited by the subjects of the present study, the average calcium requirement, computed at a 30% utilization rate, was 769 mg. in contrast to the average of 494 mg. obtained by Sherman's method. For those who were virtually in equilibrium or storing calcium (1, 2 and 76 through 95) the Sherman values are the higher, but these subjects must have utilized more than 30% of their dietary calcium in order to have been in equilibrium at such

TABLE 2

Calcium requirement of Sherman's ('20) subjects as computed by two methods

DESIGNATION OF SUBJECT		WEIGHT	CALCIUM INTAKE	CALCIUM BALANCE	CALCIUM REQUIREMENT		UTILIZATION RATE USED IN COMPUTATION [1]
					Computed on basis of 70 kg.		
In table 1 Sherman ('20)	By original investigator				By Sherman's method	By method used in this study	
		kg.	*mg.*	*mg.*	*mg.*	*mg.*	%
1, 2	Bertram (1878)	70	275	—6	285	295	30 [2]
3	Renvall ('04)	71	860	+28	820	756	22
4, 5	von Wendt ('05), subject G	72	195	—207	385	592	50
6	Holsti ('10)	58	359	—220	660	1026	33
7, 8, 9	Sherman, Mettler and Sinclair ('10)	62	465	—156	577	985	30
10–15, inc.	Sherman ('20), subject I	61	390	—140	613	980	30
16–24, inc.	Sherman, Gillett and Pope ('18), subject L	57	280	—46	409	532	30
25–34, inc. 49–55, inc.	Sherman, Gillett and Pope ('18), subject K	52	288	—88	528	782	30
35–41, inc.	Sherman, Wheeler and Yates ('18), subject M	59	140	—110	307	602	30
42–48, inc.	Sherman, Wheeler and Yates ('18), subject N	55	250	—100	450	742	30
56, 57, 58	Sherman ('20), subject E	69	323	—130	330	495	76
59–63, inc.	Sherman ('20), subject R	80	212	—114	285	518	30
64–68, inc.	Sherman and Winters ('18), subject O	54	330	—110	567	1075	23
69–75, inc.	Sherman, Winters and Phillips ('19), subject P	67	303	—171	497	910	30
76–81, inc.	Rose ('20), subject E.D.B.	54	349	+42	400	280	30 [2]
82–89, inc.	Rose ('20), subject R.S.E.	56	291	+17	416	234	30 [2]
90, 91, 92	Rose ('20), subject E.H.	45	261	+10	383	350	30 [2]
93, 94, 95 [3]	Rose ('20), subject E.W.	48	300	+82	323	28	30 [2]

[1] Calcium utilization rates could be computed for only five subjects; for the others a rate of 30% was assumed.

[2] Obviously, the rate of calcium utilization must have been greatly in excess of 30%.

[3] The data for nos. 96 and 97 could not be located in the literature.

low levels of intake.[2] Moreover, the fact that Rose's subjects (i.e., nos. 76 to 95) were able to store calcium suggests that they may have been in a condition of poor calcium nutrition prior to the beginning of the experiment; such a condition might have resulted in more efficient utilization of calcium—if Rottensten's ('38) findings on rats can be applied to man. If these individuals had utilized more than 30% of their calcium, then their true requirements would lie somewhere between the values computed by these two methods.

In the present study, the requirement for calcium has been determined from data collected while the seven subjects were ingesting a fixed dietary. The computed values, therefore, can apply to these individuals only while they are on dietaries which are similar to the one used in this study with respect to the distribution and quantity of non-milk foods and identical with it in the proportion of milk calcium to non-milk calcium. The latter consideration is important, inasmuch as there is some evidence [3] that the calcium of plant foods may not be as well utilized as is the calcium of milk. Obviously, then, if these individuals were to obtain a significantly larger proportion of their dietary calcium from non-milk sources, the quantities of calcium designated here as the minimal requirement might be insufficient. Under the conditions of this experiment the amount of milk calcium needed for equilibrium by subjects Hb, Bm, Jo, Ws, Mh, Jd and Rd, respectively, would represent 57, 62, 70, 60, 65, 71 and 76% of their total requirement. Translated into terms of fluid milk, these percentages indicate daily requirements of 236, 281, 360, 313, 353, 405 and 615 gm. in addition to the non-milk foods of the

[2] Such extraordinary balances coincident with a meager intake of calcium were never encountered during the present study; if they are the result of an adaptation to a low calcium dietary (Nicholls and Nimalasuriya, '39), the process of adjustment must have taken a relatively long time, inasmuch as Mh and Ws, who had subsisted on a calcium-poor dietary for at least 3 years prior to the beginning of the experiment (Breiter et al., '41), were unable to attain calcium equilibrium at comparably low levels of intake.

[3] Unpublished data reveal that although two of these subjects utilized as much of the calcium of carrots as they did that of milk, the other five utilized far less.

basal dietary. Practically speaking, then, six of these adults could have attained calcium equilibrium on less than 1 pint of milk daily, but, on such a milk allowance, subject Rd would have drained calcium from his skeleton at the rate of 32 mg. each day.

SUMMARY ANND CONCLUSIONS

This study reports the calcium requirement of seven adults ranging in age from 21 to 42 years. The requirement was computed from data on calcium balances secured at levels of intake almost sufficient to induce calcium equilibrium and from data concerning the extent to which each subject could utilize milk calcium. Balances of +24, +5, —39, —5, —13, —36 and —22 resulted when the respective subjects ingested 574, 568, 498, 603, 580, 451 and 873 mg. of calcium daily. Since these subjects could utilize, respectively, only 35, 31, 30, 20, 18, 15 and 20% of the calcium of milk, the following adjustments in calcium intake would have been necessary in order to bring about calcium equilibrium: —68, —16, +129, +25, +74, +235 and +108 mg. The total respective requirements, therefore, would be 506, 552, 627, 628, 654, 686 and 981 mg. calcium daily; they average 662 mg. which, when it is based on weight, height and surface area, amounts to 10.7 mg. per kilogram, 3.9 per centimeter and 391 per square meter.

These requirements, calculated on a 70-kg. weight basis, average 752 mg.—which is 67% greater than Sherman's 450-mg. requirement. But the latter figure is too low because it is predicated on the assumption that adults utilize 100% of their dietary calcium.

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FURTHER EXPERIMENTS ON THE CALCIUM REQUIREMENT OF ADULT MAN AND THE UTILIZATION OF THE CALCIUM IN MILK [1]

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The experiments reported in this paper are a continuation of those previously reported (Steggerda and Mitchell, '39) on the same subject. The same methods of study have been used and need not be repeated in detail here.

The general plan was to place each subject upon a basal diet containing on the average 203 mg. of calcium daily. The general nature of the basal diet was the same as that already described except for the use of a somewhat wider range of foods, mainly low-calcium foods, to satisfy the larger number of subjects involved. Besides the foods contained in the previous diet, the following were employed in varying amounts, either as substitutes for other foods or as additions to the diet: farina, rice, egg, liver, corn, carrots, tomato juice, apricots, peaches, bananas and lettuce. Supplements of vitamins A and D were taken daily. After 4 or 5 days on a constant intake of food, collections of feces and urine were made in successive 4-day periods until it was apparent that the subject was adjusted to this low level of calcium nutrition. Carmine was used as a feces marker.

In succeeding metabolism periods, supplements of milk products were added to the basal diet in amounts to provide

[1] The investigation was aided by grants from the Graduate School of the University of Illinois, as well as from the American Dry Milk Institute, and from the following business concerns: The Creamery Package Company, the Cherry Burrell Company, and the Manton Gaulin Company.

enough calcium for approximate body equilibrium. The milk products tested included liquid skim milk, dried skim milk ("dry milk solids"), liquid whole milk and homogenized milk. During these periods, small adjustments were made in the basal diets to equalize the energy intake in basal and test periods. All experimental periods consisted of a succession of 4-day collection periods (very occasionally 5-day periods) continued until it was evident that an adjustment to the experimental diet had occurred.

There were nine male adult subjects, consisting of graduate students and staff members, varying in age from 24 to 39 years. The ages, body weights, body heights and surface areas of the subjects are given in table 1. Throughout the experiment they remained in good health and maintained their body weights.

TABLE 1

Description of experiment subjects

SUBJECT	AGE	BODY WEIGHT	HEIGHT	BODY SURFACE [1]
	yrs.	*kg.*	*cm.*	m^2
Ha	24	69.8	186	1.93
Sp	30	72.6	175	1.82
Na	39	63.5	169	1.73
Fa	29	55.8	169	1.64
Cl	38	62.6	175	1.76
St	36	82.5	180	2.02
Fo	24	65.8	171	1.77
Ma	26	79.4	182	2.01
Ne	27	74.8	186	1.99

[1] Estimated by the DuBois height-weight formula (DuBois and DuBois, '16).

EXPERIMENTAL RESULTS

The experimental results are summarized in table 2. It seems unnecessary to report the calcium metabolism data for the individual 4-day periods. Suffice it to say that in the twenty-five experimental periods, the average standard deviation of the 4-day calcium balances about the mean balance for the period was 53 mg.

TABLE 2

The calcium metabolism data and estimates of utilization and requirement based on them

SUBJECT AND BODY WEIGHT IN KG.	CALCIUM SUPPLEMENT TESTED	TEST PERIOD	AVERAGE DAILY METABOLISM OF CALCIUM					UTILIZATION OF SUPPL. CALCIUM	INDICATED Ca REQUIREMENT:	
			Ca intake		Ca excreted		Balance		Per kg. body weight	Per m^2 body surface
			Basal diet	Suppl. food	In feces	In urine				
		days	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*%*	*mg.*	*mg.*
Ha	None	20	187	0	212	173	—198	..	...	...
69.8	Dry milk solids	24	198	543	513	193	+35	42	9.3	341
	Liquid skim milk	20	186	337	353	190	—20	53	8.0	291
	Dry milk solids	20	209	337	390	166	—10	52	8.2	293
Sp	None	20	176	0	173	180	—177	..	...	...
72.6	Dry milk solids	16	188	337	309	266	—50	36	9.4	376
Na	None	16	187	...	178	75	—66	..	...	...
63.5	Dry milk solids	20	166	337	392	110	+1	21	7.8	288
	Liquid skim milk	20	199	337	434	104	—2	18	8.6	316
Fa	None	32	165	0	168	76	—79	..	...	...
55.8	Dry milk solids	32	172	410	459	113	+10	21	9.5	324
Cl	None	28	215	0	191	146	—122	..	...	...
62.6	Dry milk solids	20	272	528	616	198	—14	18	14.0	499
St	None	20	195	0	216	82	—103	..	...	...
82.5	Liquid whole milk	16	213	431	548	105	—9	21	8.3	340
	Homogenized milk	20	213	438	558	106	—13	20	8.7	354
Fo	None	12	224	0	295	83	—154	..	...	...
65.8	Homogenized milk	16	211	435	595	85	—34	28	11.7	433
Ma	None	20	258	0	263	98	—103	..	...	...
79.4	Liquid whole milk	20	227	430	487	142	+28	33	7.2	285
	Homogenized milk	20	290	438	556	128	+44	31	7.4	292
Ne	None	16	222	0	278	52	—108	..	...	...
74.8	Liquid whole milk	21	254	430	589	67	+28	29	7.9	295
	Homogenized milk	21	245	438	609	64	+10	26	8.6	322
	Homogenized milk	20	252	420	619	69	—16	20	9.8	378
	Averages							29	9.55 [1]	357 [1]

[1] These values are the averages of the averages for the nine experimental subjects.

In the test periods of the experiment, the supplemental calcium from the milk products contributed an average of 65.5% of the total dietary calcium. The excretion of calcium occurred mainly in the feces: In the basal periods an average of only 33% of the total output of calcium appeared in the urine, while in the test periods, only 24% so appeared.

The utilization of the supplementary calcium was computed by determining its sparing effect on the excretion of body (endogenous) calcium. For example, subject Ha in his basal low-calcium period lost daily from his body 198 mg. of calcium. In the following period his calcium intake was increased by 554 mg., a supplement of "dry milk solids" accounting for 543 mg. As a result of this added intake, the loss of calcium from the body was entirely obviated and a slight storage of calcium, 35 mg. daily, brought about. Thus, the increased intake of calcium, 554 mg., spared 198 mg. of body calcium and induced a storage of 35 mg. The utilization of the supplemental calcium may be computed according to the following formula:

$$[(198 + 35) \div 554] \times 100 = 42.$$

Thus, the percentages in column 9 of table 2 measure the sparing effects of the supplemental forms of calcium with reference to the endogenous losses of calcium from the body, taking due account also of any storages of calcium, which were purposely either prevented entirely or kept at minimal levels. While considerable variation exists among these percentages of utilization, the variation seems to be the result mainly of differences in individual performance. Subject Ha was consistently high in his utilization of milk calcium, his average utilization being 49. Subject Ma was intermediate with utilizations of 31 and 33%, while subjects Na and St were consistently low. It is interesting to compare the performance of the latter subject in this experiment with his performance in the previous experiment (Steggerda and Mitchell, '39), in which he was the sole subject. In the present experiment he utilized the calcium of liquid whole milk to the extent of 21% and the calcium of homogenized milk to the extent of 20%.

In the former experiment he utilized the calcium in "dry milk solids" and in calcium gluconate to the same extent, i.e., 20%.

The results of this experiment do not afford any basis for assuming differences in the biological value of calcium among the different milk products tested. The data for subjects Ha and Na reveal no significant difference between liquid skim milk and "dry milk solids" in this respect, while the data for subjects St, Ma and Ne indicate no difference between liquid whole milk and homogenized milk. While the average utilizations for the three products, 32 for "dry milk solids," 31 for liquid milk (both whole and skim) and 25 for homogenized milk, might indicate an inferiority in the biological value of calcium in homogenized milk, the lower average for this product is largely a result of the fact that subject Ha, the highest utilizer of calcium in the group, was not used in testing homogenized milk. The average utilization of 29% seems applicable to all the milk products tested.

The calcium requirements of the subjects were estimated by computing the amount of dietary calcium required for equilibrium. Such estimations could be made only in those periods during which supplemental calcium was fed. An illustration of the method can be taken from the data of the second experimental period for subject St. In this period, an intake of 644 mg. of calcium induced an average daily deficit of 9 mg. Since the supplemental calcium was being utilized to the extent of 21%, the deficit of 9 mg. could presumably be erased by increasing the intake by 43 mg. ($9 \div .21 = 43$), making the estimated requirement 687 mg. Slight positive balances of calcium have been corrected for by deducting from the intake an amount of supplemental calcium estimated by the same method, to be the dietary equivalent of the observed balance. All estimates of calcium requirements have been expressed both in milligrams per kilogram of body weight (column 10), and in milligrams per square meter of body surface (column 11). Obviously these requirements relate to the particular sources of calcium included in the experimental diets, which were all characterized by containing milk products

in amounts to furnish about two-thirds of the content of calcium. To the extent that other sources of calcium would be differently utilized, to that extent the above calculated requirements of calcium for adult maintenance would be modified. However, since milk calcium appears to be as efficiently utilized as any form of dietary calcium, any modification of the requirements calculated above, necessitated when other diets are used, would in all probability raise the estimates. Hence, these estimates may be regarded as minimal in all probability.

The average calcium requirements are 9.55 mg. per kilogram of body weight, or 357 mg. per m^2 of body surface. The requirements are expressed in these two ways in recognition of the fact that no one method of expression has been established as superior to all others. Evidence for believing that the calcium requirement of maintenance varies with the body weight raised to a power less than 1, if not with the surface area, has been developed elsewhere (Mitchell and Curzon, '39). Expressed to the m^2 of body surface, the calcium requirements in table 2 are somewhat less variable than when expressed to the kilogram of body weight, the coefficients of variation being 19.1 and 21.6, respectively. However, the difference in variability is too small to constitute a weighty argument in favor of the former method of expression. The problem involved here is sufficiently important to merit further, and more direct, study.

DISCUSSION

The finding in these experiments that the commercial desiccation of milk does not appreciably affect the biological value of its calcium is in agreement with the results obtained in rats in a publication from this laboratory (Shields et al., '40).

The calcium requirement for adult maintenance indicated by this study, i.e., 9.55 ± 0.46 mg. per kilogram of body weight and 357 ± 15 mg. per m^2 of body surface, is considerably higher than the estimate of Sherman ('20), i.e., 0.45 gm. per 70 kg. body weight, or 6.4 mg. per kilogram. It is in good agreement, however, with the estimate of Leitch ('36–'37),

obtained from a much larger selection of published experiments, i.e., 0.55 gm. per woman subject with an average body weight of 55.6 kg., equivalent almost exactly to 10.0 mg. per kilogram of body weight. It is also in good agreement with the value of 9.75 mg. per kilogram body weight, deduced more recently by Mitchell and Curzon ('39) from a still larger selection of experiments on the calcium metabolism of adult human subjects.

In view of the importance attached to Sherman's estimate of the calcium requirement of adult man, and of the preponderate position it occupies in past and current nutritional writings, it seems necessary in evaluating the much higher figure obtained in this experiment to inquire into the cause for the great discrepancy between the two.

Sherman's estimate was derived by averaging the calcium output, reduced to a common body weight of 70 kg., observed in ninety-seven balance periods of 3 to 8 days' duration upon a much smaller, but unstated, number of adult subjects. The following quotation from Sherman's article is pertinent in this connection:

> Experiments made to test calcium requirements have sometimes involved the use of diets furnishing so little calcium that the output (although greater than the intake) has been depressed below the point at which equilibrium could actually be maintained. Largely for this reason it is probable that some of the data for indicated calcium requirement in table 1, based as they are on the data of output when the intake was somewhat insufficient, are appreciably below the true requirements of the respective subjects. Doubtless there are also cases in which the requirement was overestimated through the use of diets unnecessarily rich in calcium for the purpose of the test: but these appear to be fewer in number and the general average of 0.45 gm. of calcium (equivalent to 0.63 gm. of CaO) is probably not above the true requirement.

It is to be noted that Sherman's data of calcium output were not corrected to calcium equilibrium, as were the data of this experiment. This would, in fact, have been impossible for most, if not all, of the experiments compiled by Sherman,

since few if any of them were planned to afford information as to the percentage utilization of the calcium in the foods tested. The inability to make such a correction would not lead to serious error in the estimate of requirement if the compilation included about as many experiments in which the calcium balance was positive as experiments in which it was negative, and if the positive balances were as great on the average as the negative ones. But the data were not selected in that fashion. The large majority of the data compiled were taken from metabolism periods in which negative calcium balances were secured, and in those few periods where the balances were positive, they were comparatively small in magnitude. On the contrary, many of the negative balances were large, some even exceeding 200 mg. of calcium daily. Thus, subject R (Sherman, '20) during five consecutive 3-day periods excreted 320, 320, 310, 340, and 340 mg. of calcium daily and his calcium requirement per 70 kg. body weight is entered in the table (nos. 59 to 63 inclusive) as 280, 280, 270, 290 and 300 mg. daily. But during these periods the calcium balances were all negative to the extent of 90, 110, 110, 130 and 130 mg. daily. If the output of calcium is determined in part by the amount of calcium consumed, then to bring this subject into calcium equilibrium would require an increase in his intake of calcium which would in turn induce an increase in the calcium output. Hence, the observed calcium output does not correspond to the requirement of dietary calcium, and must in fact be considerably less than the maintenance requirement.

Sherman's method of computing calcium requirements for maintenance seems to imply that the output of calcium is independent of the intake, at least for wide ranges of intake. This in turn implies that within limits around the point of equilibrium the calcium intake is utilized completely by the human body. But the graphical presentations of the relationship between intake and output of calcium by adult human subjects as given by Leitch ('36–'37) and later by Mitchell and Curzon ('39) indicate clearly a close correlation. In the

latter case, the correlation is measured by a coefficient of +0.800 and a regression equation

$$y = 0.683x + 3.094$$

where y is the output of calcium daily per kilogram of body weight, and x is the intake similarly expressed. In the present experiment also a close relationship is indicated. Thus, in the basal periods where the diet provided only 2.93 mg. of calcium per day per kilogram of body weight, on the average, the output of calcium averaged 4.70 mg. per kilogram. In the other periods on higher levels of calcium, averaging 8.96 mg. per kilogram daily, the output of calcium was also much higher, averaging 8.95 mg. per kilogram. Considering the abnormality of the distribution of intakes in this experiment, the regression of output on intake seems best expressed by the equation

$$y = 0.687x + 2.69$$

x and y having the same significances as above defined.

It thus seems clear that Sherman's calcium requirement of maintenance in man is too low, because of the method by which it was derived, and this error in method probably accounts for the difference between his estimate and that of Leitch, of Mitchell and Curzon, and of this experiment, all of which agree well.

However, it appears that the significance of any average dietary calcium requirement, no matter how impeccable its method of derivation may be, is uncertain when applied to individual cases. Individual variability in this respect on the same type of diet seems to be extreme, as the present experiment clearly shows. Change in the sources of dietary calcium may be expected to change the dietary requirement inversely as the percentage utilization varies. Hence, any indicated requirement applies strictly only to the sources of calcium actually used in the estimation.

The common procedure of estimating the prevalence of calcium undernutrition in a population by comparing the estimated calcium content of individual or family dietaries (an estimation subject to large error) with an average re-

quirement, can yield judgments of extremely dubious validity, not only because of individual variability in requirement and of differences in the utilization of the calcium of different foods, but also because of the great adaptive powers of the human organism. It appears that in the presence of an inadequate supply of any nutrient, including calcium, the body can adjust itself to the situation, either by a more economical use of what little is available, or by a lowering of its own requirements, so that eventually it comes into equilibrium with the limited food supply. Only during this adjustment period can the body be adjudged undernourished, since only in this period is the body suffering a loss of nutriment. When adaptation is complete, the body replaces from its restricted supply all losses of the nutrient from its body, and unless some subsidiary ill effects ensue, it may reasonably be regarded as adequately supplied with food.

Instances of the adaptation of the body to low-calcium regimes are given by Nichols and Nimalasuriya ('39) in Ceylon and by Owen and Irving ('40) among the indigent of Scotland. In the latter article, instances of calcium equilibrium, or near equilibrium, were observed on intakes of 3 to 4 mg. of calcium daily per kilogram of body weight to which the subjects had become accustomed over a period of years. These subjects were beyond middle life and many of them showed evidences of osteoporosis (x-ray examination), which probably developed during the period of adjustment to the low level of calcium intake occasioned by their economic status. An earlier study by Owen ('39) may be interpreted in much the same way, although the calcium restriction was not so severe and the data are not so complete with reference to the description of the subjects. The observations of Basu, Basak and Sircar ('39) on three adult subjects in India consuming vegetarian diets based on rice and whole wheat, very low in their calcium content, indicate for two of the subjects, a successful adaptation to this restricted calcium supply.

Possibly all these cases, even after successful adjustment to the low intake of calcium, are not in the best condition of

health—certainly their dietary is not one that can be recommended—but their calcium balance sheets afford no evidence of malnutrition or no suspicion that malnutrition will eventuate. Nor can their nutritive state be appraised by the mere comparison of their consumption of calcium with an average requirement deduced from balances on experimental subjects that have not experienced a long period of subsistence on diets containing these low quantities of calcium. In such cases objective evidence of malnutrition or ill-health should be forthcoming before an adverse judgment is passed.

SUMMARY AND CONCLUSIONS

Twenty-five calcium balance periods, extending over 12 to 32 days each, were carried out on nine adult men. The first period on each subject involved the testing of diets containing only an average of 203 mg. of calcium daily, but otherwise complete. In subsequent periods the basal diet was supplemented with milk products (liquid whole milk, liquid skim milk, "dried milk solids" and homogenized milk) in amounts to provide enough calcium for approximate equilibrium. Comparisons of the data secured in basal and test periods permitted the estimation, first, of the percentage utilization (biological value) of the supplemental calcium for maintenance, and, second, of the requirement of dietary calcium for equilibrium, i.e., the calcium requirement of maintenance.

No evidence was obtained that the calcium in the different milk products studied was utilized with different degrees of efficiency. An average utilization of 29% was observed, although considerable individual variability existed in this respect, the individual averages being, in increasing order, 18, 19, 21, 21, 25, 28, 32, 36 and 49. In particular, it seems clear that the commercial desiccation of milk, or its homogenization, does not appreciably impair the biological utilization of its calcium.

The average calcium requirement for equilibrium (maintenance) was 9.55 ± 0.46 mg. daily per kilogram of body weight, or 357 ± 15 mg. per m^2 of body surface. This requirement

relates to diets in which about two-thirds of the calcium content is provided by milk products. It agrees well with the estimate of Leitch ('36–'37) and that of Mitchell and Curzon ('39), deduced from compilations of published data, but it is 50% higher than the estimate of Sherman ('20).

Sherman's estimate of the calcium requirement of maintenance in man is too low by reason of the method of derivation, which neglects to consider the close positive correlation between the intake and the excretion of calcium.

The dangers attending the use of any average calcium requirement of maintenance in assessing the prevalence in a community or population of calcium undernutrition are discussed.

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THE DISTRIBUTION OF PYRIDOXINE (VITAMIN B_6) IN MEAT AND MEAT PRODUCTS [1]

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In previous reports from this laboratory values for the thiamine (Mickelsen, Waisman and Elvehjem, '39 a), riboflavin (Mickelsen, Waisman and Elvehjem, '39 b), pantothenic acid (Waisman, Mickelsen and Elvehjem, '39) and nicotinic acid (Waisman, Mickelsen, McKibbin and Elvehjem, '40) content of meat and meat products have been given. In this paper we wish to report values for the pyridoxine content of animal tissues.

Studies of this kind appear especially advisable in view of recent reports which claim the successful use of this vitamin in treating pellagrins that do not respond completely to the thiamine, riboflavin and nicotinic acid therapy (Spies, Ladisch and Bean, '40; Spies, Bean and Ashe, '39). A definite response has also been observed in the macrocytic anemia of pellagrins and in pernicious anemia patients in relapse (Vilter, Schiro and Spies, '40). Smith and Martin ('40) have reported its use in the treatment of cheilosis. The availability of the crystalline vitamin simplifies the bioassay procedure and allows the values to be expressed in terms of the pure pyridoxine.

The biological assay methods thus far reported (Lunde and Kringstad, '38; Schneider, Ascham, Platz and Steenbock, '39; Wilson and Roy, '38; György, '35; Dimick and Schreffler, '39;

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Halliday and Evans, '37) are based on the cure of acrodynia or upon a combination of growth and relief of symptoms in rats. Edgar, El Sadr and Macrae ('38) have described an assay based on growth responses. Complete cure of acrodynia is not effected by pyridoxine alone, but at least one other factor, the "essential fatty acids," is concerned in curing the syndrome (Birch, '38; Schneider, Steenbock and Platz, '40). Furthermore, in most cases the diets used were incomplete since suboptimum growth was obtained even with the administration of the vitamin B_6 concentrate or crystalline material. Further work has since demonstrated that previous rations were low in a number of factors, the most outstanding of which was pantothenic acid. A basal ration developed in this laboratory (Conger and Elvehjem, '41) has been shown to be adequate in all respects except for vitamin B_6. When the crystalline vitamin is added at levels of 2–6 μg. per day, the growth response is proportional to the amount of crystalline material.

EXPERIMENTAL

The method of assay used is essentially that of Conger and Elvehjem ('41). The basal ration has the following percentage composition: purified casein [2] 18; sucrose 75; salts IV [3] 4; corn oil [4] 3. Each 100 gm. of this mixture contained the following: thiamine 200 μg.; riboflavin 300 μg.; nicotinic acid 2.5 mg.; choline 30 mg.; and calcium pantothenate [5] 1 mg.; fullers earth filtrate of liver conc. 1:20 ≅ 4% original liver powder; and haliver oil 2 drops weekly.

The fullers earth filtrate was prepared by dissolving 100 gm. of liver concentrate powder 1:20 in 100 ml. of distilled water and shaking with 500 ml. of butanol in a mechanical

[2] Labco casein, Borden and Company, New York City.

[3] Phillips and Hart (J. Biol. Chem., vol. 109, p. 657, 1935). Salt mixture with $MnSO_4$: 4 H_2O increased from 0.7 to 10 gm. per kilogram of salt mixture.

[4] Corn oil is added to the remainder of basal ration only in amounts sufficient to last 1–2 weeks and rations are kept at low temperature in the dark to prevent rancidity.

[5] Merck's.

shaker for 2 hours. After the two layers separated, the butanol layer was removed and 500 ml. more butanol added. This extraction with butanol was continued until five extractions had been made. When anhydrous butanol was used, it was found necessary to add approximately 25 ml. of water following the second or third extraction to prevent the water layer from becoming too viscous for effective extraction. The combined extracts were concentrated under reduced pressure to a syrup at which point water was added and concentration continued to remove the last traces of butanol. The residue was taken up in 100 ml. of distilled water and adjusted to pH 1–2. Five grams of English fullers earth was then added to adsorb out the vitamin B_6. After shaking for 20 minutes the fullers earth was removed by filtration through a thin pad of coarse filter cel. The pad was washed with 10–15 ml. of N/10 HCl. The adsorption on fullers earth was repeated four more times using 5 gm. portions of the fullers earth each time. The combined filtrate and washings were neutralized to pH 7 with NaOH and concentrated under reduced pressure. The concentrated filtrate was dried on the casein of the ration at a level equivalent to 4% of the original liver concentrate. Water solutions of the vitamins were spread on the casein and dried in a current of warm air at 50°C. The salts and sucrose were then added and the entire ration was finely ground.

Male albino rats weighing 35–40 gm. at 21 days of age were used throughout the assays. The rats were placed in individual cages and fed the basal ration for a 2-week depletion period. An average gain of 10 gm. during the first week and 7 gm. during the second week of the depletion period was obtained. The average gain of control rats continued on the basal ration was 5 gm. per week for a 5-week period. At the end of the 2-week depletion period the standard controls were fed the basal ration to which crystalline vitamin B_6 had been added at levels of 50, 75 and 100 μg. per 100 grams of ration. The rats on the standard amounts of pyridoxine were included in each series of assays to eliminate possible individual differences. More than thirty rats on the crystalline pyridoxine showed

remarkable uniformity in growth responses to the three levels of the vitamin throughout the assay period of 5 weeks. Conger and Elvehjem ('41) have demonstrated that the ration is complete in all factors except vitamin B_6 and that growth is the best criterion for measuring the pyridoxine potency.

The meats, which were prepared for assay according to methods previously described by Mickelsen, Waisman and Elvehjem ('39 a) were mixed in the basal ration. Four to eight rats were used to obtain the pyridoxine values for a particular sample. The rats were weighed and examined at weekly intervals. No acrodynia or other gross symptoms were observed in the control rats or in the animals receiving the supplement during the assay period. At the end of a 5-week assay period the vitamin B_6 content of the meats was calculated by comparing the growth of the rats on the meat supplement with the growth of the rats receiving crystalline pyridoxine (vitamin B_6 hydrochloride). Preliminary work showed that when calculations were based on the percentage of meat in the ration, more consistent results were obtained than when food consumption records were used.

RESULTS

The results of our bioassays are shown in table 1. The values are expressed in micrograms per gram of fresh and dried meat. Kidney is a good source of vitamin B_6. Two samples of beef kidney and two samples of pork kidney showed approximately the same content of the vitamin. With the exception of the beef liver sample, livers of the various species contained less than 15 μg. per gram of dried tissue. Lamb liver was slightly higher than pork liver and veal liver. Muscle was a moderately good source of pyridoxine. Three samples of pork ham contained 20–28 μg. per gram dry weight. Three samples of pork loin showed a vitamin B_6 content of 14–19 μg. per gram of dried loin. Beef muscle was only slightly lower than veal muscle. Lamb muscle fell in the same range. Pork and beef heart had a somewhat lower vitamin B_6 potency than other muscle meats. A single sample of dark chicken showed

TABLE 1

Pyridoxine content of meat and meat products

SAMPLE	SAMPLE NUMBER	VITAMIN B_6 HYDROCHLORIDE (μG. PER GRAM)	
		Dry	Fresh
Beef brain	77	5.3	—
Beef heart	73	11.2	2.4
Beef kidney	126	21.3	4.3
Beef kidney	81	19.5	4.4
Beef kidney (stewed)	112	14.4	—
Beef liver	131	25.0	7.3
Beef lung	138	3.5	0.7
Beef muscle	128	16.2	3.8
Beef muscle	105	14.5	4.0
Beef muscle (fried)	106	12.0	—
Beef pancreas	113	7.5	2.0
Beef spleen	130	5.7	1.2
Beef tongue	82	4.2	1.2
Chicken (dark)	66	< 8	< 2
Lamb muscle	80	11.8	3.0
Lamb liver	96	12.6	3.7
Pork ham (dried)	95	24.6	6.0
Pork ham (dried)	124	28.2	6.7
Pork ham (dried)	139	21.7	5.5
Pork ham (fresh)	140	23.5	5.9
Pork ham (fried)	141	15.7	—
Pork ham (roast)	142	12.1	—
Pork ham (fried)	52	17.6	—
Pork ham (boiled)	115	10.6	—
Pork ham (smoked)	116	9.8	2.9
Pork ham (tender)	72	7.7	2.4
Pork ham (tender)	119	8.8	2.9
Pork ham (tender)	117	11.0	3.8
Pork loin	125	18.8	5.1
Pork loin	89	19.5	6.5
Pork loin	36	14.5	4.5
Pork loin (roasted)	94	11.5	—
Pork kidney	83	18.1	4.0
Pork kidney	137	34.8	7.0
Pork heart	104	15.6	3.5
Pork liver	86	9.9	3.2
Pork liver	122	10.3	3.3
Veal liver	97	10.3	3.0
Veal muscle	129	16.2	4.0
Veal muscle	134	19.4	4.2
Veal muscle	103	16.3	4.4
Frozen fillet of cod	135	19.6	3.4
Frozen salmon steak	136	21.2	5.9
Yellow corn		4.8	—
Wheat germ		15.9	—
Winter milk		—	1.3

a low vitamin B_6 content, although the levels fed were too low to ascertain exactly how much pyridoxine was present. Beef tongue, beef spleen, beef pancreas and beef brain were in the range of the lower potencies. Beef lung was the poorest source of the vitamin. Since fish muscle had been reported to be a good source of the "anti-acrodynia" factor by György ('35), Wilson and Roy ('38), and Lunde and Kringstad ('38), we assayed samples of cod and salmon. The samples were obtained in the frozen state and dried in the usual manner. The results indicated that fish muscle is indeed a good source of vitamin B_6 containing about the same as mammalian muscle.

Determinations made on processed hams showed a marked reduction in the pyridoxine potency as a result of the processing. The boiled ham sample contained approximately half that of pork ham, and smoked ham showed a similar decrease. Three samples of tender ham showed a loss of about 60% of the vitamin B_6 as a result of the tenderizing process. Stewed beef kidney and fried pork retained two-thirds of their original potencies. Pork loin showed a reduction of almost half in the roasting process. A single fresh pork ham was divided into four portions in order to further ascertain the loss of the vitamin during household cooking. These portions were fed fresh (sample 140), dried (sample 139), fried (sample 141) and roasted (sample 142). The results obtained by assaying these samples showed that the drying process resulted in no significant loss of vitamin B_6 potency, while frying resulted in about 30% loss and roasting in 45% loss. A slight loss was also noted during the frying of beef muscle as shown by the vitamin B_6 content of the parallel samples 105 and 106 which were from the same animal. The loss of vitamin B_6 during various cooking processes is probably the result of leaching since this vitamin is known to be heat stable and very soluble in water when in the free form.

A single sample of yellow corn contained 4.8 μg. per gram, a value less than any meat assayed except beef tongue and beef lung. This is contrary to the early report of Copping

('36) which stated that corn was a good source of vitamin B_6. Wheat germ was found to contain almost as much pyridoxine as most muscle meats. Winter milk showed a potency of 1.3 μg. per milliliter. It is difficult to compare the values we obtained for the pyridoxine content of meats with the few values reported for the "antiacrodynia" potency of meats reported by György ('35) and Schneider, Ascham, Platz and Steenbock ('39). As yet the only report of the vitamin B_6 content of meats using a chemical method was one in which Swaminathan ('40) reported 13.4 μg. per gram for fresh sheep liver and 4.5 μg. per gram for fresh sheep muscle. The value for muscle is about the same as we obtained, 3.0 μg. per gram fresh, but his value for liver is considerably higher than ours. There is good agreement between our value for milk of 1.3 μg. per milliliter and Swaminathan's of 1.7 μg. per gram.

A previous report (Mickelsen, Waisman and Elvehjem, '39 a) has demonstrated that pork muscle is a better source of thiamine than liver, whereas the assays for riboflavin (Mickelsen, Waisman and Elvehjem, '39 b), pantothenic acid (Waisman, Mickelsen and Elvehjem, '39) and nicotinic acid (Waisman, Mickelsen, McKibbin and Elvehjem, '40) have shown that liver is invariably superior to muscle tissue as a source of these factors. The assays reported here, however, indicate that muscle tissue must be considered a better source of vitamin B_6 than liver. It should be emphasized that considerable variation in the B_6 content of a given tissue may be encountered if several samples are examined. However, assay of a few samples of each type of tissue gives values which allow a comparison of one meat with another and with other foodstuffs.

Although there are no accurate figures available on the human requirement for pyridoxine, it may be possible to approximate the amount necessary from the human requirements of other vitamins and comparative growth experiments with rats using the same vitamins. It has been shown by work in this laboratory (Arnold and Elvehjem, '38; Waisman,

Henderson and Elvehjem, '40) that 100 μg. of thiamine and 200 μg. of riboflavin per 100 gm. of diet will produce optimum growth in the rat. The level of pyridoxine necessary to give maximum growth in the rat lies between these levels of the other vitamins, but closer to that of thiamine. Since the daily human requirement for thiamine is about 2 mg. per day (Cowgill, '39; Sherman and Lanford, '39), it appears that the daily requirement of pyridoxine would also be about 2 mg. Using this figure, meat is able to furnish a large part of this requirement since the average vitamin B_6 content of muscle tissue is 5–6 μg. per gram of the fresh meat. This means that 300–400 gm. of fresh muscle meat is necessary to supply the daily need of pyridoxine.

SUMMARY

1. The pyridoxine content of meat and meat products has been determined by means of a rat growth method.

2. Kidney and muscle were the richest sources containing 20–30 μg. per gram while heart and liver were somewhat lower with 10–15 μg. per gram; spleen, pancreas, brain and lung were poor sources containing less than 8 μg. per gram on the dry basis.

3. The fried meat samples showed least destruction, but roasting and stewing caused losses of from 20 to 50%. Commercial processing of various meat samples resulted in similar losses.

4. Some samples of fish muscle, milk, and cereals were also assayed.

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SODIUM AND POTASSIUM SALTS IN THE TREATMENT OF EXPERIMENTAL DIABETES AND DIABETES MELLITUS [1]

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ONE FIGURE

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The relation of sodium and potassium salts to carbohydrate metabolism has been sporadically investigated for a number of years. Beard ('18) studied the effect of unrestricted salt intake on diabetic patients, especially from the point of view of possible kidney damage. In the course of his paper, he mentions that only one patient on high sodium chloride, and one on high sodium bicarbonate showed increased sugar tolerance. He observed that his studies allowed no deductions concerning the utilization of carbohydrates during the period of high sodium chloride intake. Glass and Beiless ('30) reported beneficial effect of intravenous injections of hypertonic sodium chloride solution on the blood sugar in diabetes.

The subject received new attention when McQuarrie, Thompson and Anderson ('36) gave large doses of salt (60 to 90 gm. daily) to a series of diabetic children. Four patients, 13 to 15 years old, were intensively studied in a metabolism ward. A basal diet with known very low potassium and sodium values was used throughout the experiments. These authors claimed

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Acknowledgment and thanks are due Dr. Bertnard Smith for permission to study patients in the Cedars of Lebanon Hospital, Dr. Herman Zeiler for laboratory cooperation, Dr. Paul Greeley for operative assistance, and Dr. A. L. Chaney for chemical methods.

that following the ingestion of these large doses of sodium chloride, blood pressure rose, glycosuria lessened, fasting blood sugar levels were lower, the R.Q. increased, and insulin reactions were more frequent. The blood pressure and the glycosuria returned to previous level when the sodium chloride was stopped. Potassium acted in just the opposite manner, and when sodium and potassium were balanced in the McQuarrie basal diet, no noticeable change occurred. MacLean ('35) reported similar results in a diabetic patient at the Mayo Clinic. Sanstead ('36) gave doses of sodium chloride varying from 10 to 90 gm. to eight male adult diabetics, and in all his patients he found a lowering of blood sugar. A reduction of insulin or an increase in diet was made possible by this high ingestion of sodium chloride. His protocols do not indicate the effect on glucose excretion, nor is there any reference to the ratio of sodium and potassium.

EXPERIMENTS ON PATIENTS

Our first experiments were carried out in a series of twelve patients in the diabetic ward of the Los Angeles County Hospital without extensive preliminary metabolism studies. These patients were stabilized and without changing the diet were then given doses of sodium and potassium chloride varying from 20 to 30 gm. daily. We found that larger doses usually could not be employed without causing nausea. Blood sugar, glycosuria, and blood pressure were carefully followed. Critical analysis of the data from these experiments failed to reveal any constant or marked change in the patient's status which could definitely be attributed to the change in therapy. Occasionally minor changes in glucose excretion were noted after the administration of sodium chloride or of potassium chloride, but these changes were not constantly in the same direction. For example, in any one patient carried through several experimental periods the administration of sodium chloride was followed at one time by decreased glucose excretion and later by increased excretion. The same variability in results was

noted in the experiments with potassium chloride. An illustrative protocol of the experiments on human subjects is presented in table 1.

TABLE 1

Effect of increasing sodium intake on glucose excretion in a diabetic patient[1]

DATE	INTAKE			URINE		BLOOD		
	Carbohydrates	Sodium	Glucose	Sodium	Potassium	Sugar	Serum sodium	Serum potassium
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*mg./100 cc.*	*mg./100 cc.*	*mg./100 cc.*
5–24	173	0.921	19.09	1.405	0.842			
5–25	173	0.921	37.32	0.890	0.841			
5–26	173	0.921	43.15	1.44	1.03	290	329	18
				Sodium period				
5–27	179	8.771	39.12					
5–28	179	8.771	36.70	3.03	0.995			
5–29	182	12.721	42.2	9.70	1.51			
5–30	182	12.721	32.21	8.77	1.72			
5–31	182	12.721	41.35	8.16	1.62			
6– 1	182					238	332	19

[1] It should be noted that the "en seal" tablets of sodium chloride used in this experiment contained a small amount of carbohydrate which accounts for the differences in carbohydrate intake.

The data here presented were obtained from one patient, a 14 year old girl who was placed on the McQuarrie diet for a period of 11 days during which time the insulin was adjusted to allow a fairly constant glucose excretion. The diet contained 173 gm. carbohydrate, 70 gm. protein, 89 gm. fat, 0.921 gm. sodium, 1.963 gm. potassium. For the first 2 days 20 gm. of sodium chloride were given daily and 30 gm. daily thereafter. Insulin was constant for the experimental period at 10-0-10 units regular insulin. Certainly in this patient no effect of 20 or 30 gm. of sodium chloride on glucose excretion could be demonstrated.

At this stage it was apparent that our investigations were not demonstrating the uniform pattern suggested by previous authors. Varying and inconclusive results provided considerable contrast to the almost perfect score of McQuarrie et al. and of Sanstead. It seemed then logical to assume that if sodium and potassium were important in diabetic therapy,

some other factor than the mere addition of either ion to the diet was necessary to explain the action.

Experiments were projected on various animal species. Our preliminary tests on rats indicated that no definite change in sugar excretion followed alterations in potassium and sodium intake, and for this reason no further studies were undertaken. Technical difficulties caused us to eliminate the rabbit. The goat was in many respects a satisfactory experimental subject, and we shall later present the protocols on some of our investigations on this animal.

EXPERIMENTS ON DOGS

Our experiences, like those of other students of carbohydrate metabolism, have indicated that the dog is an excellent subject for experiments of this nature. Female dogs were depancreatized and given enough food and insulin to keep the weight of the animal constant and still have a definite but not unduly large glycosuria. Then the animals were not used until at least a month after operation and only at a time when they had completely recovered and were in good condition. After the control period an experimental period of 3 days was run under identical conditions except for the addition of sodium or potassium chloride to the diet. This time period was soon found to be inadequate and was increased to 5 days and still later to even longer periods. The dogs' food was divided into two equal portions and fed at 8 A.M. and 5 P.M. daily. Protamine Zinc Insulin was injected subcutaneously immediately after each feeding. The dogs usually ate their food within a few minutes, but whenever it was necessary to coax the dogs to eat, the food was always consumed within ½ hour. The animals were kept in metabolism cages which were cleaned daily. Urine collections for a 24-hour period were made at 8 A.M. Venous blood for blood sugar determinations was taken immediately before feeding. Blood for serum determinations of sodium and potassium was always taken before the 5 P.M. feeding. The methods used for chemical analyses were as follows: Blood sugar, Folin-Wu; urine sugar, Benedict's

quantitative; urine and serum sodium, Hoffman adapted by Chaney; urine and serum potassium, Brown, Robinson, and Browning adapted by Chaney.

In the first experiment a diabetic dog subsisted on a constant diet of meat, pancreas and sugar. This contained 100 gm. of carbohydrate and between 1.3 and 2.2 gm. of potassium per day. This animal was observed during seven 5-day periods, arranged so that the control periods alternated with the experimental. In two of the experimental periods 2.3 gm. extra sodium were given, and in one period 4 gm. extra potassium. In both instances the salts given were chlorides. We found that the animal excreted an average of 43 gm. of glucose per day during the first control period, but during all the remaining periods the average daily excretion varied between 22 gm. and 25 gm. In other words, the glucose excretion remained practically constant during the last six periods, and suggests that the fall from the initial period was the result of spontaneous improvement.

TABLE 2

Effects on glucose excretion of increases of potassium and of sodium intake in the diabetic dog. The basal diet was different in parts A and B

	A				B			
	I Control	II Potassium	III Control	IV Sodium	V Control	VI Sodium	VII Control	VIII Sodium
No. days	16	9	33	5	15	8	6	6
Average glucose output (gm.)	11.08	25.07	14.31	7.64	18.39	23.10	32.50	26.60
Sodium intake (gm.)	0.611	0.611	0.611	4.84	0.544	3.696	0.044	3.190
Average sodium output (gm.)	0.641	0.650	0.618	4.190	0.511	4.777	0.032	2.98
Potassium intake (gm.)	0.900	4.030	0.900	0.900	0.561	0.561	0.561	0.561
Average potassium output (gm.)	0.800	3.74	0.739	0.316	0.754	0.470	0.310	0.736
Average of daily blood sugars (mg./100 cc.)	250 a.m. 183 p.m.	560 a.m. 275 p.m.	500 a.m. 260 p.m.	520 a.m. 260 p.m.	420 a.m. 250 p.m.	416 a.m. 269 p.m.	400 a.m. 242 p.m.	

An attempt was then made to duplicate the results obtained by McQuarrie et al., using the diet of milk, eggs and sucrose as employed by them in their studies on children. Choline hydrochloride (100 mg. daily) was also used in the dog experiments to replace pancreas. This is necessary to prevent fatty degeneration of the liver.

As will be seen by examination of table 2A, the average daily glycosuria increased during the high potassium period and decreased during the high sodium period. Further examination of these apparent results, however, is deemed necessary. The increase in glucose output during the potassium period over that of the control period is certainly marked. It was considered advisable to extend the subsequent control period to 33 days because during this time, despite a rigidly constant regimen, the glycosuria was steadily decreasing. We have charted successive subdivisions of this period, and figure 1 shows this definite trend toward diminishing glycosuria. The chart illustrates the spontaneous changes which may occur in the status of diabetic animals. During this period had we in

Fig. 1 Shows variations in glucose excretion of a diabetic dog under constant conditions. Besides the ordinary day-to-day fluctuations there is to be noted a definite improvement (beginning on the twentieth day) in the diabetic condition during the later days. This illustrates the "spontaneous changes" which may occur in diabetic animals despite the maintenance of rigidly constant conditions.

any way changed the experimental conditions, we might easily have been misled into drawing erroneous conclusions. During the last 13 days of this control period, the dog was excreting only an average of 6.63 gm. of glucose daily. Then, upon the addition of sodium chloride to the diet (to give an average intake of 4.84 gm. of sodium daily) the glycosuria actually increased over the last few days of the preceding control period.

Because of intestinal disturbances, resulting from the high salt administration, it was now necessary to adopt a new diet. In order to keep the potassium intake at a minimum, a diet of pancreas (which is very low in potassium) and sucrose was substituted. As will be noticed upon examination of this experiment in table 2B, there are no consistent results. In the 29 days comprising the first three periods a gradual increase in glycosuria occurs even though sodium chloride has been given for 8 days between two control periods. The final period of increased sodium chloride intake is associated with a slightly diminished output of glucose. We do not see any definite indications of a sodium effect in this experiment.

A second depancreatized dog gave equally negative results when carried through the same type of experiment. During a period of 8 days, with a constant diet of pancreas and sucrose containing carbohydrate 80 gm., sodium 0.044 gm., and potassium 0.561 gm. daily, the average daily glucose excretion was 2.13 gm. During the next period of 19 days the carbohydrate and sodium were kept the same and the potassium was increased to 3.7 gm. per day. The average sugar excretion remained practically the same, 1.98 gm. During the next control period (5 days) the carbohydrate intake was increased to 150 gm. per day and the sodium to 0.544 gm. per day, with the potassium 0.561 gm. per day. The average sugar excretion was 26.2 gm. During the fourth period (7 days) with the carbohydrate and potassium the same, the sodium intake was increased to 3.96 gm. per day, and the average sugar excretion was 12.44 gm. per day.

EXPERIMENTS ON A GOAT

The depancreatized goat is able to utilize approximately 80% of the carbohydrate ingested without insulin, and at the same time maintain its weight. The diet consisted of sucrose and alfalfa. Weighed amounts of alfalfa were given the goat daily, and the part not eaten was weighed. Because the same amount of food was not eaten each day we have substituted the term "per cent of glucose utilized" for the phrase "glucose excretion" used in previous tables. The sugar solution, or the sugar solution plus the added sodium or potassium chloride, was taken at the same time daily, 8.30 A.M., but the alfalfa was eaten throughout the day. All blood chemical determinations were made on venous blood drawn between 3.30 P.M. and 4.00 P.M. Any attempt to substitute a food lower in potassium for the alfalfa resulted in the death of the animal. The potassium content of alfalfa is about 2%. The protocols show no marked effect on the carbohydrate utilization following the addition of sodium or potassium to the diet. The results of an experiment on a diabetic goat are found in table 3. This experiment on the goat may be of importance because, in the presence of considerable glycosuria, variations of sodium and of potassium intake were followed by definite changes of excretion of these ions, yet were without any significant influence on the excretion of sugar or on the blood sugar.

TABLE 3

Effects on glucose excretion of increases of potassium and of sodium intake in the diabetic goat

	I Control	II Sodium	III Control	IV Potassium	V Control	VI Sodium
No. days	4	5	5	5	12	8
Average glucose output (gm.)	55.9	64.9	57.5	47.1	67.3	57.0
Glucose utilization (%)	79.81	77.2	77.8	81.2	74.2	79.6
Sodium intake (gm.)	0.76	2.51	0.62	0.55	0.66	3.00
Average sodium output (gm.)	0.530	1.98	0.40	0.54	0.178	2.38
Potassium intake (gm.)	10.00	9.30	7.80	10.80	8.50	8.80
Average potassium output (gm.)	13.4	10.2	10.5		11.02	11.08
Average of daily blood sugars (mg./100 cc.)	160 p.m.	189 p.m.	250 p.m.	267 p.m.	252 p.m.	259 p.m.

We studied the diabetic goat during another experiment lasting 27 days during which time the animal was fed as before but given 8 units of insulin per day. The time was divided into four periods consisting of two control periods interposed between a high sodium and a high potassium period. Here, as before, variations of sodium and of potassium intake were followed by corresponding changes of excretion of these ions without any change in the average daily urinary glucose or in the "per cent utilization of carbohydrate".

SUMMARY

Our experiments on human subjects indicate that there is no constant response to the ingestion of sodium chloride which warrants a conclusion as to its therapeutic value in diabetes mellitus. Limited experiments with potassium likewise gave inconclusive results. In some patients an apparent change in carbohydrate metabolism following the ingestion of sodium or of potassium is suggested, but this phenomenon is not sufficiently constant to justify any sound conclusions.

Studies on diabetic animals definitely show that ingestion of sodium chloride or of potassium chloride may be followed by changes in sugar excretion of such magnitude as to suggest causal relationship. However, these changes are not constant. In the same animal, under experimental conditions identical as far as can be determined, the ingestion of sodium chloride is followed at one time by diminished glycosuria and at another time by increased glycosuria. Contrasting experiments on two dogs, the same paradoxical results are noted. Detailed analyses of our tables indicate that in some instances the results are more apparent than real and may actually represent coincidental changes in status.

Our results do not entirely agree with those reported by McQuarrie, Thompson and Anderson. Differences in conditions of the experiments may to some extent account for this. Their experiments were conducted on children to whom they gave very large doses (60 to 90 gm.) of salt; most of our work was done on adult diabetics or on the depancreatized animal,

and we were unable to employ the large salt dosage used by McQuarrie et al., without producing nausea, vomiting, or diarrhea. The most convincing experiment published by them concerned a salt craver who showed definite decrease in sugar output when sodium chloride was given. In no other case are their results as conclusive: the change in sugar output following the ingestion of sodium chloride or of potassium chloride suggests causal relationship, but may also be interpreted as a change in status of the patient. The possibilities of such changes influencing conclusions from therapeutic experiments in diabetes are explored and extended in our discussion of figure 1.

The experiments on the goat seem of particular interest in that large changes in mineral equilibrium were produced by our experiments without demonstrable corresponding changes in sugar metabolism.

It is probable that changes in cellular and tissue metabolism not elucidated by our protocols may explain the results. Our human and animal studies suggest one conclusion: changes in carbohydrate metabolism which follow ingestion of sodium or potassium chloride cannot be explained as a result of the mere addition of these salts to the dietary intake.

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REQUIREMENT OF THE MOUSE FOR PANTOTHENIC ACID AND FOR A NEW FACTOR OF THE VITAMIN B COMPLEX [1]

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TWO FIGURES

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Pantothenic acid has been shown to be required by the chick (Jukes, '39; Woolley et al., '39), the rat (Hitchings and Subbarow, '39; El-Sadr et al., '39; Oleson et al., '39), the dog (McKibbin et al., '40), and by man (Spies et al., '40). The instability of pantothenic acid to hot alkali has become an important factor in the investigation of this vitamin. Pennington, Snell and Williams ('40) have inactivated pantothenic acid in a yeast extract by autoclaving for 30 minutes with 0.5 N sodium hydroxide at 15 pounds pressure. McKibbin and his co-workers ('40) were able to destroy pantothenic acid in a liver extract by heating at 87–93°C. for 2 hours in the presence of 0.2 N potassium hydroxide.

In this paper, data will be presented which indicate that the mouse requires pantothenic acid and one or more factors contained in the alkali-stable portion of the filtrate fraction of the vitamin B complex.

EXPERIMENTAL

The diets used had the following percentage composition: purified casein, 30; Osborne and Mendel salt mixture, 7; sucrose, 55; hydrogenated cottonseed oil,[2] 5; cod liver oil, 3.

[1] Presented in part at the meeting of the American Chemical Society at Detroit, September, 1940.

[2] Crisco.

Ten milligrams of riboflavin, 5 mg. of thiamine chloride, and 5 mg. of vitamin B_6 hydrochloride were added per kilo of diet. To this basal diet (diet B) alkali-treated liver extract was added, in an amount equivalent to 50 gm. of liver extract [3] per kilo of diet (diet B-L).

The alkali treatment of the liver consisted in heating on the steam bath for 1 hour, with occasional stirring, a solution of 100 gm. of 92% alcohol soluble liver extract in 500 cc. of water, to which 20 gm. of sodium hydroxide (to make 1 N) had been added. The solution was allowed to cool, and made just acid to litmus with sulfuric acid.

Albino weanling mice, 21–25 days old and bred in our laboratory, were placed in individual wire-mesh cages and fed a diet free from water-soluble vitamins (diet-B without addition of vitamins), for a period of 5 days. They were then fed the experimental diets. Those animals receiving the pantothenic acid supplement were injected subcutaneously with 40 micrograms daily (6 days per week) of d-calcium pantothenate [4] in water solution.

The pantothenic acid requirement was determined by injecting varying amounts of d-calcium pantothenate daily (6 days per week) into each of five litter-mate groups, each group consisting of eight animals. They were given diet B. The data presented in figure 1 show that under the experimental conditions present the mouse requires about 30 micrograms of d-calcium pantothenate daily.

Mice receiving the unsupplemented basal diet (diet B) grew for a short time, after which they exhibited only maintenance (fig. 2). After 20–30 days, they showed symptoms of filtrate factor deficiency, characterized by loss of hair in one or more areas of the body, which turned into complete alopecia in the later stages. They also developed a serous exudate around the eyes, spinal curvature, and a kicking twitch of the hind legs. The animals finally lost weight and died on about the

[3] Supplied by the Wilson Laboratories, Chicago, Ill.

[4] The d-calcium pantothenate was generously supplied by Merck and Company, Rahway, N. J.

fifty-fifth day. A diet containing yeast or whole liver extract protected completely against this deficiency.

When the basal diet (diet B) was supplemented with 40 micrograms daily (6 days per week) of d-calcium pantothenate, the animals grew at a rapid rate (fig. 2), and showed no signs

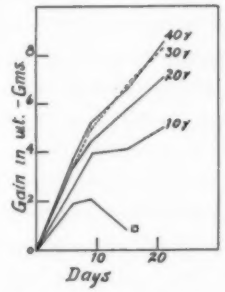

Fig. 1 Growth of mice receiving increasing amounts of d-calcium pantothenate daily (6 days per week). The animals were fed diet B. Each curve represents the average for eight mice.

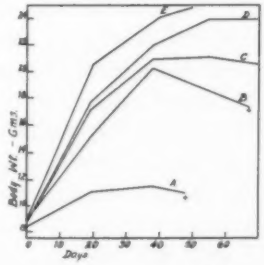

Fig. 2 Average growth curves of mice receiving various fractions of the vitamin B complex. Curve A shows the growth of animals receiving only diet B, and curve B that of animals receiving diet B-L. Curve C shows the growth response of mice receiving diet B plus 40 micrograms daily (6 days per week) of d-calcium pantothenate, and curve D that of mice receiving diet B-L plus the same amount of d-calcium pantothenate. Each curve represents the average for eight animals. For comparison curve E showing the average growth of mice fed upon our stock diet has been included. The difference between the growth rate of the control animals (curve A) and that of the control animals shown in figure 1 is due to individual variations, and appears to depend on the reserves of the missing factors in the young mice.

The (+) indicates the time at which the first animal died.

of deficiency until about the fortieth day. At this time the coat became shaggy, and the animals stopped growing.

Mice receiving the alkali-treated liver extract in addition to thiamine, riboflavin, and vitamin B_6 (diet B-L), did not grow as well as animals on diet B plus pantothenic acid, and showed signs of deficiency at an earlier date. By the eighty-sixth day, one-half of the animals were dead.

When diet B-L was supplemented with calcium pantothenate, the animals grew at a rate which was close to normal, and in the majority of cases appeared in good condition. Some of these animals, however, presented an unclean appearance similar to that shown in the beginning stages of filtrate factor deficiency. The reason for this has not yet been ascertained.

These experiments have been repeated, using the butanol-soluble portion of the 92% soluble liver extract [5] as a source of the filtrate fraction of the vitamin B complex, with similar results.

These observations indicate that in addition to thiamine, riboflavin, and vitamin B_6, the mouse requires pantothenic acid and an alkali-stable member of the filtrate fraction of the vitamin B complex. Our findings are in agreement with those reported for the rat by Dimick and Lepp ('40) and by Unna ('40). This unknown factor may be identical with Elvehjem's factor W. Experiments conducted in our laboratory [6] have shown that mice which have ceased to gain in weight after being kept on a diet containing thiamine, riboflavin, nicotinic acid, and vitamin B_6, will increase in weight at a rate close to normal if they are given a daily dose of a factor W concentrate (prepared according to Black et al., '40). Whether this response is due to the pantothenic acid present in the concentrate alone, or to the combined effects of pantothenic acid and some unknown factor, are points which still require investigation.

[5] Prepared according to the initial step in the preparation of factor W concentrate (Black et al., '40).

[6] Unpublished data.

DISCUSSION

The possibility of the existence of a second alkali-labile factor is not excluded by these experiments. Animals receiving untreated liver extract show a better growth and appear to be in better condition than those receiving alkali-treated liver extract plus pantothenic acid, and they behave similarly in all respects to animals kept on our stock ration. Whether this difference is due to the presence in liver extract of an alkali-labile factor distinct from pantothenic acid, or to some other cause, cannot be decided from these experiments. McKibbin, Black and Elvehjem ('40) have recently reported a series of experiments on dogs, using an inactivated liver preparation similar to that used in our experiments. On the evidence of these experiments they suggest the existence of an alkali-labile factor, distinct from pantothenic acid, which is required by the dog.

Woolley ('40 a, '40 b) has reported that inositol, or the salt of its phosphoric ester, phytin, is required by the mouse as a growth and "anti-alopecia" factor. According to his experiments, mice receiving all the known crystalline components of the vitamin B complex in addition to yeast extract, ceased to grow in about 4 weeks, and developed marked loss of hair within 6 weeks. Unless the new factor was given, these animals died within 2–3 weeks from the onset of symptoms.

In our experiments, no case of marked alopecia has been observed among fifteen mice which have received only thiamine, riboflavin, vitamin B_6 and pantothenic acid, and these animals are still alive after 140 to 185 days on the experiment. Our basal diet, however, differs from that used by Woolley in that it contains more protein, less sucrose, more fat, no yeast extract, and especially a larger amount of pantothenic acid.

Norris and Hauschildt ('40) have reported that mice require a water-soluble factor other than thiamine, nicotinic acid, vitamin B_6, riboflavin and the "filtrate factor." In the absence of this factor from the diet, mice were found to develop skin lesions similar to those which we have observed

in filtrate factor deficiency, but which, according to our observations, are prevented in every case, and can be cured, by pantothenic acid. Here again, our diets differ in composition from that used by these workers.

To what extent the discrepancy between our results and those reported by Woolley, and by Norris and Hauschildt, can be explained by the differences in the rations used or in other experimental conditions, remains to be determined.

Our findings indicate that for mice, as has been reported for rats, four components of the vitamin B complex, namely, thiamine, riboflavin, vitamin B_6, and pantothenic acid, provide an almost adequate supplement. The deficiency of the unknown factor shows itself mainly in the suboptimal growth, but the animals survive for more than 6 months.

SUMMARY

It has been shown that besides thiamine, riboflavin, and vitamin B_6, at least two additional factors are necessary for normal growth in the mouse. One of these is pantothenic acid. Under the conditions present in our experiments, the daily requirement of the mouse for this factor is about 30 micrograms in terms of d-calcium pantothenate. The second of these is alkali-stable and is present in liver extract.

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IRON METABOLISM IN HUMAN SUBJECTS ON DAILY INTAKES OF LESS THAN 5 MILLIGRAMS [1]

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TWO FIGURES

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The requirement of the human for iron is undisputed but the amount required is controversial. The pendulum of recommendations for daily intakes has swung from a high of 17 mg. (Leverton and Roberts, '37) to a low of 5 mg. (Farrer and Goldhamer, '35) but has rested for the most part between 12 and 15 mg. of iron (Sherman, '32, '37).

Recent developments in the field of research have challenged any interpretation of requirements which is based upon determining the relation of the intake to the excretion of the metal and using this relation as a measure of the body's need for iron.

McCance and Widdowson ('38) have presented substantial evidence for their theory that the gastrointestinal tract has no ability to regulate by excretion the amount of iron in the body. This means that the iron found in the feces is that which has not been absorbed rather than iron which has been absorbed, utilized, and re-excreted. It follows then that the iron excreted in the feces is not a criterion of the body's utilization or need but rather a reflection of its absorption, and many factors other than need may influence absorption.

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[2] With the technical assistance of Lila Meyerott, Emily Shepard, Grace Otis and Maxine Armstrong.

In addition to this changed viewpoint much has been done in the last few years to direct attention to the great theoretical and practical significance of serum or plasma iron. Several investigators, Moore, Doan and Arrowsmith ('37), Heilmeyer and Plotner ('37) and Baer ('38) have shown that serum iron is of metabolic importance in the mammalian organism because it serves as a medium for iron transportation. Of particular value to the study of iron metabolism is the evidence presented by Moore et al. ('39) and Hahn et al. ('38) to show that the amount of iron being absorbed from the intestinal tract is indicated by increases in the plasma or serum iron.

With these facts in mind, namely, that the intestine probably does not excrete iron and iron excretion, therefore, is not a measure of utilization, and that changes in serum iron values may indicate iron absorption, the present study was planned for three, and later four college girls. The first objective was to follow the fecal excretion of iron on a diet which contained as little iron as possible and yet could be considered adequate in every other respect. It was of particular interest to see if the fecal excretion of iron would ever significantly exceed the intake, regardless of how low the intake became. The second objective was to follow the serum or transport iron values on this low iron intake in an effort to study the relationship of the level of intake and excretion to endogenous iron metabolism. Later a third objective developed, namely, to study the effect of substituting meat for the milk in the basal diet.

PROCEDURE

The plan of the study was to follow, over a period of several months, the excretion of iron by young women on a constant diet optimum in all known dietary essentials except that it contained less than 5 mg. of iron, and to make closely associated blood tests which might reflect changes in the iron content of the body as a result of such a low iron intake.

The subjects were four healthy college girls who will be referred to as subjects A, B, C and D. Subject A was 21,

subjects B and C were 22, and subject D was 28 years old at the beginning of the study.

Subjects A, B and C were on the metabolism regimen continuously for 94 days from September 19 to December 23, 1939. On January 4, 1940, subjects A and B returned to the regimen, subject A for 90 days until April 8th, and subject B for 150 days until June 2nd. Subject C was studied again for 25 days in March and subject D for 75 days from the middle of March until June. This totals 622 days of study on four individuals.

The study was arbitrarily divided into consecutive 5-day periods which numbered 1 to 49, inclusive. A basal dietary was arranged for each 5-day term and repeated period after period. The menu for each of the 5 days was different but the food for each 5-day period was identical in kind, amount, and order of use. A few foods of negligible iron content per serving, puffed rice, angel-food cake, white sugar, butter and whipping cream, were permitted ad libitum to meet individual caloric requirements. Since only foods which were low in iron could be used, beef and many fruits and vegetables were excluded. This resulted in a monotonous though not unpalatable diet which required ingenuity to prepare and a scientific viewpoint to consume month after month.

Every effort was made to secure foods of uniform composition. Most of the food was purchased at one time. Individual portions of perishable foods were weighed into wax paper cups and stored in a freezing locker; peaches and tomatoes were canned with distilled water in glass containers; vegetable soup and applesauce [3] were secured in glass containers; the milk [4] and citrus fruit were sampled daily for analysis.

Certain special precautions were taken in order to avoid increasing the iron content of the diet by contamination from the usual sources. All food preparation was done in Pyrex oven or flameware,[5] all china and cooking utensils were rinsed

[3] Given by Beech-Nut Packing Company.

[4] Dairy products were given by Roberts Dairy, Lincoln.

[5] Given by the Corning Glass Works.

in distilled water and the girls used distilled water entirely for food preparation and drinking.

The diet furnished daily: 67 gm. of protein, 1.2 gm. of calcium, 1.5 gm. of phosphorus and 3.5 mg. of iron. A vitamin concentrate [6] containing 6200 U.S.P. units of vitamin A, 900 U.S.P. units of D, 75 International units of B_1, 200 International units of C, and 20 Sherman units of G (riboflavin) was taken daily together with 1 mg. of thiamine chloride.[7] In order to insure that iron would be the only limiting factor in the diet, a supplement of copper sulfate solution to provide 2 mg. of copper daily was also taken.

Changes were made in the basal dietary from time to time in an effort to determine the relation of such a change to the blood picture and to the excretion of iron. During periods 32, 33, 34, 116 gm. of lean beef [8] replaced the 750 ml. of milk daily. This quantity of meat contained the same amount of protein as the milk. During the next three periods, 35, 36 and 37, 232 gm. of meat were used daily while the milk and part of the egg white and cottage cheese were omitted to keep the protein level of the diet constant. Since the iron in the serum and the amount of iron excreted changed when the subjects were on the meat regimen, it was repeated during periods 47, 48 and 49 when 116 gm. of meat were again used daily in place of 750 ml. of milk.

Collections of all stools, urine, and menses were made during the entire study.

The following materials were made into smooth brown digests with 20% hydrochloric acid and then adjusted to a known volume: (1) the composite samples of feces for each 5-day period, (2) the individual samples of foods, (3) the mixtures of the daily samples of milk and citrus fruit taken as representing the 5-day periods, and (4) the menses. A 25 ml. aliquot of the brown digest was pipetted into a 300 ml. Kjeldahl flask and the flask placed in a Pyrex fume duct.

[6] [7] Given by Abbott Laboratories.

[8] Supplementary financial aid was given by the National Livestock and Meat Board.

Concentrated nitric acid was used to digest the easily oxidizable material and perchloric acid for that which was difficult to destroy. The resulting salt solution was boiled with water or 2% sodium sulfite to remove any excess perchloric acid. The solution was filtered and made to a final volume of 100 ml. In this solution iron was determined as the thiocyanate by the method developed by Stugart ('31). A Sanford-Sheard Photelometer, which had been calibrated with solutions of known iron concentration, was used for making the color comparisons. The analytical work was done in a laboratory especially designed and equipped to preclude iron contamination during the preparation and analysis of the materials.

Certain blood determinations formed an important part of this study. Hemoglobin content together with red and white cell counts was determined each week. For these 0.5 ml. of venous blood was taken from subjects A, B and D and capillary blood from a finger prick from subject C. About once a month the iron content of the blood serum and the cell volume were determined and smears made for differential leucocyte and reticulocyte counts. At this time 35 to 50 ml. of venous blood were taken from each subject except subject C.

The methods as set forth in "Clinical Diagnosis by Laboratory Methods" by Todd and Sanford ('37) were followed closely for hemoglobin, cell counts, and cell volume. Hemoglobin was converted into oxyhemoglobin in 0.1% sodium carbonate solution and read in the aforementioned Photelometer. The method used for the serum iron determinations was that developed by Moore and Arrowsmith ('37). The blood was collected in paraffined tubes and centrifuged for 45 minutes. Tenth normal HCl was added to ionize the iron and then the protein was precipitated with 20% trichloracetic acid. The deproteinized serum was digested with H_2SO_4 and H_2O_2. In the resulting clear solution, iron was determined as the thiocyanate.

RESULTS

The data for the entire study are summarized in table 1, to show the total intakes and losses of iron for each of the

TABLE 1

Intake, loss and balance and blood data of four subjects at different levels of iron intake

SUBJECT	DAILY DIET	PERIOD	NO. DAYS	AVERAGE DAILY INTAKE OF IRON	TOTAL MG. OF IRON						HEMOGLOBIN GM./100 ML.		R. B. C. M/CU.MM.	
					Intake	Losses				In venous blood samples	Begin	End	Begin	End
						Fecal exer.	Urinary exer.	Menstrual loss	Total loss	Balance				
				mg.										
A	Low-iron	1–19 inc.	94	3.06	287.64	287.64	6.06	29.40	323.10	— 35.46	67.17	15.0	13.7	5.07	4.03
	Low-iron	20–31 inc.	60	3.09	185.40	213.00	4.26	16.40	233.66	— 48.26	95.31	14.3	13.6	4.13	4.43
	Low-iron plus 116 gm. beef	32–34 inc.	15	6.54	98.10	54.45	1.38	4.00 [1]	59.83	+ 38.27	23.45	13.4			4.23
	Low-iron plus 232 gm. beef	35–37 inc.	15	9.17	137.55	93.60	2.02	4.00	99.62	+ 37.98		14.2	13.7	4.47	4.57
B	Low-iron	1–19 inc.	94	3.41	320.54	281.06	5.92	46.72	333.70	— 13.16	69.04	15.0	14.5	5.41	4.31
	Low-iron	20–31 inc.	60	3.09	202.20	187.80	3.76	26.70	218.26	— 16.06	94.97	14.3	13.5	4.38	4.34
	Low-iron plus 116 gm. beef	32–34 inc.	15	6.76	101.40	54.45	1.28	5.10 [1]	60.83	+ 40.57	22.94	12.9			4.22
	Low-iron plus 232 gm. beef	35–37 inc.	15	9.58	143.85	78.75	1.57	5.10 [1]	85.42	+ 58.43			13.4		4.42
	Low-iron	38–40, 42–46 inc.	40	4.46	178.40	144.00	3.08	11.60	158.68	+ 19.72	21.94	13.3	13.8	4.48	4.26
	Low-iron plus 120 mg. iron as $FeSO_4$	41	5	124.41	622.05	330.00	0.45	....	330.45	+291.60					
	Low-iron plus 116 gm. beef	47–49 inc.	15	6.47	97.05	58.50	1.37	7.80 [1]	67.67	+ 29.38	22.78	13.5			4.37
C	Low-iron	1–19 inc.	94	3.34	313.96	313.02	6.76	27.08	346.86	— 32.90	40.24	15.0	12.9	5.03	4.01
	Low-iron	33–37 inc.	25	3.96	99.00	95.75	1.91	8.40	106.06	— 7.06		13.2	13.0	4.34	4.05
D	Low-iron	35–42, 45–46 inc.	50	4.45	222.50	187.50	4.00	66.40	257.90	— 35.40	46.57	13.8	12.6	4.66	4.13
	Low-iron plus 116 gm. beef	47–49 inc.	15	6.43	96.45	62.55	1.25	12.20	76.00	+ 20.45	22.28	13.3	12.5	3.98	4.08
						Average daily									
All A, B and D	Low-iron		517	3.50					3.83	— 0.33					
	Low-iron plus 116 gm. beef daily		60	6.55					4.41	+ 2.14					

[1] One-half of the menstrual loss for the current cycle.

subjects at the different levels of iron intake. The values for the iron content of the urine were calculated from the results of analyses of the composite samples for the first ten periods for each subject.[9] The iron loss from venipuncture was calculated from the amount of blood withdrawn and its hemoglobin content at the time. This loss is not included in the total of usual losses but placed in a separate column beside the figures showing the relation or balance between intake and usual losses. The hemoglobin and red cell values at the beginning and end of each dietary regimen are also given.

Several significant facts are emphasized by the figures in table 1. There were seven different groups of periods when the subjects were on the low-iron diet for periods varying in length from 25 to 94 consecutive days. During this time the intake was never large enough to cover the total of the fecal, urinary and menstrual losses. Considering all the subjects for this time, 517 metabolism days, the intake and total loss calculated on average daily basis, were 3.50 mg. and 3.83 mg. respectively. The negative balance, 0.33 mg. iron daily, would indicate that this amount was being used from the body stores of the metal.

The figures for the daily metabolism of subjects A, B and D when they were eating beef, have been summarized in table 2,

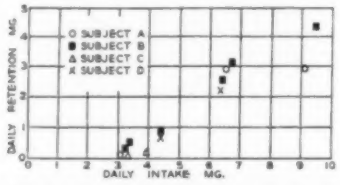
TABLE 2

Average daily iron intake and excretion during 15-day periods of beef intake

SUBJECT	BEEF INTAKE	IRON INTAKE	IRON EXCRETION	IRON RETENTION	
	gm.	*mg.*	*mg.*	*mg.*	*%*
A	116	6.54	3.63	2.91	44.49
B	116	6.76	3.63	3.13	46.30
	116	6.47	3.90	2.57	39.72
D	116	6.43	4.17	2.26	35.15
Average		6.55	3.83	2.72	41.42
A	232	9.17	6.24	2.93	31.95
B	232	9.58	5.25	4.33	45.25
Average		9.37	5.74	3.63	38.60

[9] The urinary excretion of iron under normal conditions has been found by many workers to be small and constant. This has been so consistently borne out in this laboratory that following analysis on ten urine composites for each subject, iron determinations in urine were discontinued.

which shows the per cent of the iron as well as the number of milligrams which were absorbed during these periods. The average daily intake for the three subjects for the 60 days when 116 gm. of beef were used was 6.55 mg. of iron while the total fecal, urinary and menstrual loss averaged 4.39 mg. of iron. Although figures are insufficient to show whether or not an intake of 9 mg. would promote better absorption than an intake of 6.5 mg. there is no question but that absorption was greater on a daily intake of 6.5 mg. than on intakes ranging from 3.0 to 4.5 mg. The relation of size of intake of iron to the amount absorbed is shown graphically in figure 1.



RELATION OF LEVEL OF INTAKE TO AMOUNT OF IRON RETAINED

Figure 1

After the first 94 days subject C was not returned to the basal diet but given 120 mg. iron daily as ferrous sulfate in addition to a self-chosen diet for 6 weeks. Her intake, fecal excretion, and retention of iron during this time are shown in table 3. After retaining a total of 2206 mg. during a 6-week period the iron supplement was discontinued and after 5 days she was put on the low-iron diet for 25 days, periods 33–37, inclusive. During this time on an average daily intake of 3.96 mg. she retained 0.13 daily.

TABLE 3

Absorption of iron on a daily supplement of 120 mg. from ferrous sulfate

SUBJECT	MG. IRON ABSORBED PER WEEK						TOTAL	PER CENT OF INTAKE [1]
	1	2	3	4	5	6		
							mg.	
C	533	487	606	192	94	294	2206	40.4
D	615	334	226	362	394	222	2153	39.4

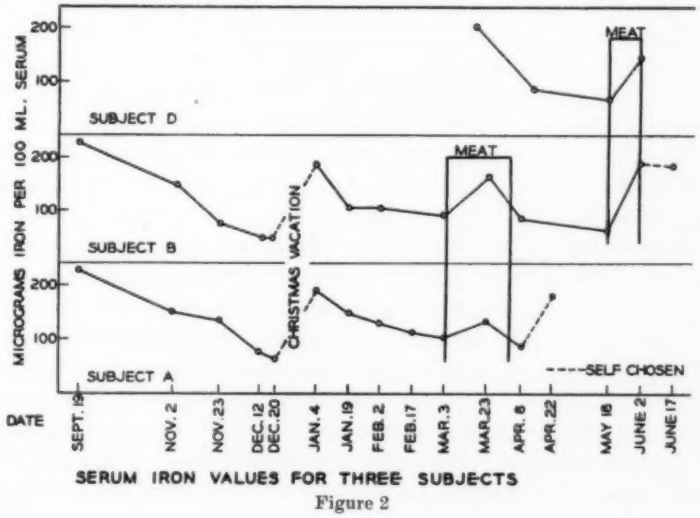
[1] Daily diet was assumed to contain 10 mg. iron.

The treatment of subject D was similar to that just described for subject C. At the end of 6 weeks of iron therapy she had retained a total of 2153 mg. of iron (table 3), and on the low-iron diet she retained 0.70 mg. daily.

When subjects B, C and D ate self-chosen diets after the metabolism study, the feces were kept for analysis for 1 or 2 weeks. Subject B had an average daily fecal excretion of 7.70 mg. of iron the first week and 9.37 mg. the second week; subject C excreted 12.05 mg. of iron daily the first week and 10.69 mg. the second; and subject D excreted 9.52 mg. daily the first week.

Throughout the entire study the results of the white cell, differential leucocyte and reticulocyte counts and the determination of cell volume remained within the limits of normal variations, and therefore reflected no measurable effects of either the low iron diet or its supplements.

Probably the most significant results of the study are the values obtained for the iron content of the blood serum of the subjects at intervals during the 8 months. These are charted in figure 2. They show a gradual and consistent drop



SERUM IRON VALUES FOR THREE SUBJECTS

Figure 2

in the serum iron during the low-iron regimen and a rise when the diet was self-chosen and when the iron content of the diet was increased by the use of beef.

At the beginning of the study the serum iron value for subject A was 227 μg. per cent and decreased to 65 μg. per cent at the end of 92 days. Subject B started with a value of 228 μg. per cent and this dropped to 50 μg. per cent in the 92 days. During 13 days of vacation, on a self-chosen diet, these values rose sharply to 190 and 189 μg. per cent for subject A and B respectively. Following vacation they again decreased and continued to decrease as the time on the low-iron diet increased. Their response was very similar to that in the fall when the study was begun. Thus, in the fall after 65 days on the low-iron diet the serum iron value of subject A had dropped 89 μg. per cent and it dropped 88 μg. per cent in the 59 days from January to March. Similarly, for subject B there had been a decrease of 92 μg. the first 59 days following Christmas vacation. Because of the difficulty of a venipuncture serum iron determinations were not made on subject C except at the end of the 94 days on the low-iron diet. At that time it was 81 μg. per cent. The serum value of subject D on a self-chosen diet and after she had taken 120 mg. iron daily for 6 weeks was 211 μg. per cent. After 25 days on the low-iron diet it had dropped to 88 μg. per cent and after 61 days to 68 μg. per cent.

The effect of adding beef as a source of iron in the diet was reflected similarly in the iron content of the blood serum of all three subjects, A, B and D. The serum iron value of subject A was determined after 59 days on the low-iron diet and was found to be 102 μg. per cent. The next 19 days she was given beef, 116 gm. daily for 15 days and 232 gm. for 4 days. At the end of this time her serum level had risen to 135 μg. per cent of iron. After 5 days on the low-iron diet her serum iron value had decreased to 87 μg. per cent, but after the next 14 days while she was on a self-chosen diet, it rose to 180 μg. per cent. The serum level of subject B before the meat was given was 97 μg. of iron and after 19 days on the

meat, 116 gm. daily for 15 days and 232 gm. for 4 days, it had risen to 162 μg. per cent. After 5 days on the low-iron diet her serum iron was 84 μg. per cent and at the end of 45 days it was 65 μg. per cent. At this point she was again given 116 gm. of beef daily for 15 days which increased the serum iron to 190 μg. per cent. This was followed by 15 days on a self-chosen diet which gave a value of 188 μg. per cent. After 2 months on a self-chosen diet her serum iron level was 220 μg. per cent which was comparable to the 228 μg. per cent it had been 8 months earlier when the study was begun. The serum iron value for subject D was 68 μg. per cent after 61 days on the low-iron diet and after 15 days of eating 116 gm. beef daily this value had risen to 144 μg. per cent. It was not possible to make determinations later on a self-chosen diet.

DISCUSSION

Discussion of the results will be limited to the relation of intake to excretion of iron, the significance of blood serum iron values on different levels of iron intakes, and the application of these to the interpretation of iron requirement.

Considering the entire study, with the omission of subject A, it appears that the intestine normally does not excrete iron and it follows that the fecal excretion of this metal would not exceed the intake over any period of observation long enough to counteract variations due to intestinal motility. If this is true, the size of fecal excretion cannot be used as a criterion on which to base dietary recommendations. Although the fecal excretions on the intake of approximately 6.5 mg. of iron were almost the same as those on 3.5 mg. this relationship does not continue as the intake increases to 9 mg. for here the fecal excretion increases to 5 and 6 mg. and on self-chosen diets to anywhere from 7 to 12 mg.

Certain additions to the basal diet may explain the negative iron balance of subject A during periods 20–31 inclusive when her daily intake and excretion averaged 3.09 mg. and 3.55 mg. respectively. During this time subject A digressed slightly from the basal diet. Since her fecal excretions never exceeded

4.9 mg. of iron daily and her serum iron values continued to decrease, the digressions were not considered of sufficient magnitude to require the omission of the metabolism figures from this report. They are not included, however, in the generalizations.

A marked decrease in hemoglobin or red cell content was not to be expected on the low-iron dietary since it has been shown that in case of a dietary restriction of iron, the depots of iron are probably used before hemoglobin production suffers. The slow response or resistance to change of hemoglobin level during the study may have been a reflection of the optimal protein and vitamin intake, or more especially the copper supplement.

Though the decrease in serum iron from the time of one determination to the next for the subject on this study did not always exceed the normal variation of 41 μg. per cent reported by Moore, Minnich and Welch ('39) the total change was always greater. For instance, the response of subjects A, B and D when changing from a self-chosen diet to the low-iron regimen involved decreases of 162, 178, and 143 μg. per cent respectively in 92 days for A and B and 61 days for D.

The decrease in serum iron values was not paralleled with a decrease in the rate of absorption of iron for as the study progressed the iron absorbed from the constant low intake did not decrease. This continued decrease in serum values is not exactly in keeping with the concept of Moore, Minnich and Welch ('39) that a state of equilibrium exists between the iron being absorbed, the adequacy of the iron stores, and the rate of hemoglobin synthesis. The total metabolism figures showed a negative iron balance on the low intakes, and a slight consistent decrease in hemoglobin values accompanied the low serum iron concentrations but iron absorption was not correspondingly affected. Apparently the iron in the serum is more labile than that which is stored and when iron demands are not met with corresponding intakes, the drain on the serum iron is greater than its replenishment from the storage depots.

The present concept of serum iron together with the results of this study suggests the possibility of conducting iron metabolism studies by determining serum iron values during different levels of iron intake. If the iron deficit or balance between intake and total loss is indicated by the iron content of the serum there would be no need for collection and analysis of feces, urine, and menstrual blood.

These data may contribute to the understanding of the iron requirement of normal women. When all the periods on the low-iron diet for all the subjects are considered, the average daily intake was 3.50 mg. of iron and the average daily total of fecal, urinary and menstrual loss was 3.83 mg. This indicates that a daily intake of 3.50 mg. of iron was not sufficient to cover the losses and that iron was being drawn from body tissues or blood. This is further substantiated by the fact that on this intake the blood serum iron consistently decreased from the upper to the lower limits of normality, and that the hemoglobin and red cell values also decreased, consistently though not strikingly.

From a practical standpoint a daily intake of 3.5 mg. of iron is seldom encountered. Even the most haphazardly chosen diet, unless made up almost solely of refined cereals, will contain more than this amount of iron. It is of scientific importance, however, to realize that an optimal intake of all known dietary essentials except iron can fail to meet the requirements of the female body. In other words, there still exists a need for iron which cannot be met by other dietary constituents.

Increasing the daily intake of iron of three of the subjects to 6.55 mg. by the use of 116 gm. of beef completely changed their metabolic picture and this level of intake might be considered adequate for these subjects. The average daily total loss of 4.41 mg. was only slightly more than the 3.83 mg. on the lower intake, an average daily retention of 2.14 mg. of iron occurred, and the serum iron values rose significantly.

Although the results may not definitely establish the adequacy of 6.5 mg. of iron daily for young women in general,

the finding that 6.5 mg. provided 2 mg. daily in excess of the total loss of these subjects indicates that this level of intake would still allow for individual variations in amount of menstrual loss, rate of intestinal motility, variations in food composition and losses in cooking.

The results of the entire study suggest that there is greater need for emphasis on a diet liberal in other minerals, protein, and vitamins than on iron as a single item. Contrary to previous interpretations of iron metabolism data, it appears that the amount of iron may not warrant special consideration in dietary practices or recommendations but that an ordinary mixed diet adequate in the other known essentials would supply ample iron for maintenance and replacement in the normal young woman.

SUMMARY AND CONCLUSIONS

The iron metabolism of four healthy college girls was studied for periods varying from 3 to 8 months. The greater part of the time the subjects were on a diet adequate in all known dietary essentials except that it contained only 3.5 mg. to 4.5 mg. of iron.

A fecal excretion of iron which did not exceed the iron intake did not mean the body was in iron equilibrium. At all times on the low-iron diet the total of fecal, urinary and menstrual iron losses exceeded the intake so that the body was losing iron from its storage depots. The extent of this loss averaged 0.33 mg. of iron daily on an intake of 3.50 mg.

When the iron content of the diet was increased to 6.55 mg. by replacing the 750 ml. of milk in the basal diet of three subjects with 116 gm. of lean beef, there was an average daily retention of 2.14 mg. of iron.

The iron content of the blood serum decreased consistently and significantly during the low-iron regimen but whenever the intake of iron was increased the serum iron levels rose markedly and then dropped again when the low iron intake was resumed.

The results suggest that there is no need for emphasis on amounts of iron exceeding 6.5 mg. daily in the diet of normal young women; emphasis should rather be placed on liberal amounts of other dietary essentials.

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CHOLINE METABOLISM

VII. SOME DIETARY FACTORS AFFECTING THE INCIDENCE AND SEVERITY OF HEMORRHAGIC DEGENERATION IN YOUNG RATS

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ONE FIGURE

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A dietary deficiency of choline and of the "labile methyl supply" produces in young rats a severe pathological state which has been named hemorrhagic degeneration. The prevention of the characteristic renal lesions and of the deposition of excess liver fat is a satisfactory test for choline and other sources of labile methyl, such as, methionine and betaine. Previous papers have considered the effect in these experiments of the age and sex of rats (Griffith, '40 a) and of supplements of cystine (Griffith and Wade, '40) and of cholesterol (Griffith, '40 b). The present report is concerned with the influence of the deficiency of labile methyl on the rate of growth and on the recovery process in surviving animals and with the relation between the food intake and the occurrence of the degenerative condition. In addition, the effect of certain dietary factors has been investigated, preparatory to using the production and prevention of hemorrhagic degeneration in an extensive study of the relation of the labile methyl supply to the metabolism of choline.

EXPERIMENTAL

The experimental procedures were similar to those previously described (Griffith and Wade, '39). The appearance of the kidneys was noted at the end of the experimental period

and the per cent of animals in each group showing the hemorrhagic state (Griffith, '40 a) is recorded in tables 2 to 5 under the heading "renal lesions". Determinations were made of liver and kidney weights and of the total chloroform-soluble substances in the liver. The term liver fat in tables 3 and 5 refers to this fraction. The values shown in figure 1 for non-protein nitrogen (Koch and McMeekin, '24), amino-acid nitrogen (Folin, '22) and glucose (Shaffer and Somogyi, '33) were obtained by the analysis of tungstic acid filtrates of pooled blood from groups of four or more rats.

Young male rats, 20 days of age and 25 to 34 gm. in weight were used in the experiments recorded in table 4. Rats, 21 to 26 days of age and 38 to 42 gm. in weight, were used in the experiments recorded in figure 1 and in tables 2, 3 and 5. The basal diets are described in table 1. The stock diet consisted of a commercial dog food.[1]

TABLE 1

Composition of diets

	AA[1]	AC 39[1]	AC 32	AC 50	AC 51	CC 3
	%	%	%	%	%	%
Amino acid mixture[2]	24	0	0	0	0	0
Purified casein	0	18	15	18	18	15
Gelatin	0	0	0	0	0	3
Cystine	0	0	0.3	0	0.3	0.1
Cholesterol	0	0	0	0	0	0.5
Lard	20	20	9	20	20	20
Sucrose (commercial)	48.9	54.9	62.6	48.9	48.6	49.3
Dried brewer's yeast	0	0	6	6	6	6
Salt mixture[3]	4	4	4	4	4	4
Calcium carbonate	1	1	1	1	1	0
Agar	2	2	2	2	2	2
Natola[4]	0.1	0.1	0.1	0.1	0.1	0.1

[1] Water soluble vitamins supplied by daily oral administration of 0.02 mg. of thiamine chloride, 0.02 mg. of riboflavin, 0.02 mg. of pyridoxine and 0.5 mg. of nicotinic acid.

[2] Prepared according to McCoy and Rose ('37) except that methionine and cystine were omitted.

[3] Hawk and Oser ('31).

[4] Fortified fish liver oil.

[1] Purina dog chow.

RESULTS

Effect of hemorrhagic degeneration on rate of growth. Young rats fed low choline and low labile methyl food mixtures develop fatty livers within 48 hours and hemorrhagic lesions within 3 to 6 days. The renal pathology is most severe between the sixth and eighth days and either results in the death of the animal or in a spontaneous recovery from the hemorrhagic state. During this critical stage the animal is noticeably sick and there is a spectacular rise in the nonprotein nitrogen of the blood. Figure 1 shows the daily change in weight of rats

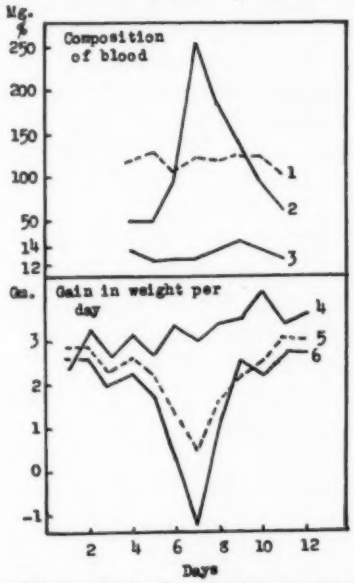

Fig. 1 The effect of hemorrhagic degeneration on the composition of blood and on the rate of growth of 40-gm. male rats. The analysis of blood, collected after decapitation, was made on pooled samples from individual groups of four or more rats maintained on diet AC 32 for 4 to 11 days. The daily changes in body weight were determined in other groups continued on the diet for 12 days.

Curve 1. Glucose of blood.

Curve 2. Nonprotein nitrogen of blood.

Curve 3. Amino-acid nitrogen of blood.

Curve 4. Average daily gain in weight of a control group of thirteen rats fed diet AC 32 plus 0.3% choline chloride.

Curve 5. Average daily gain in weight of a group of twenty-two rats fed diet AC 32.

Curve 6. Average daily gain in weight of twelve of the more severely affected rats in the group represented in curve 5.

fed diet AC 32, with and without added choline, and also the levels of blood glucose, amino acid nitrogen and nonprotein nitrogen in similar groups fed diet AC 32 for 4 to 11 days. The rapid decrease in the nonprotein nitrogen and the restoration of growth are noteworthy features of the recovery phase. Similar observations were made by Cox, Smythe and Fishback ('29) in an investigation of the toxic effect in rats of a dietary supplement of cystine.

Rats which survived the acute stage were continued on experimental diets in order to determine the extent of the recovery process. Table 2 shows results obtained with diets

TABLE 2

The effect of hemorrhagic degeneration on rate of growth of 40-gm. male rats

GROUP	DIET	EXPERIMENTAL PERIOD	NO. OF RATS [1]	FINAL WEIGHT	WEIGHT AS PER CENT OF BODY WEIGHT		RENAL LESIONS
					Kidneys	Liver	
		days		*gm.*			%
1	AC 32	7	23–21	56	1.55	7.05	96
2	AC 39	7	18–16	50	1.77	6.82	100
3	AC 32	10	27–21	60	1.80	7.45	100
4	AC 39	10	26–17	45	2.28	7.67	100
5	AC 39, 7 days + AC 32, 3 days	10	32–17	50	2.12	7.54	100
6	AC 39 + 0.3% choline chloride	10	15–15	61	1.01	4.38	0
7	AC 32	30	34–26	145	0.96	8.80	50
8	AC 39, 7 days + AC 32, 23 days	30	25–11	131	0.97	9.80	100
9	AC 39, 7 days + AC 32, 3 days + Stock diet, 20 days	30	43–21	134	1.15	5.24	88
10	AC 32 + 0.3% choline chloride	30	10–10	153	0.88	4.80	0
11	Stock diet	30	19–19	140	0.91	4.30	0

[1] Second figure shows the number of surviving rats.

AC 32 and AC 39. The latter was used in order to produce greater damage to the kidneys than was possible with AC 32. Groups 1 to 5, table 2, show the 7- and 10-day effects of the two diets, effects which are prevented by choline (group 6). The average daily food intakes during the first and second 5-day periods were 5.4 and 3.3 gm. for group 3 (diet AC 32) and 4.5 and 1.4 gm. for group 4 (diet AC 39). This decrease in food consumption during the second half of the experi-

mental period was prevented by choline (group 6), the values for the two 5-day periods being 4.5 and 5.4 gm. for this group. The more severe effect obtained with diet AC 39 is believed to be due to the omission of supplies of labile methyl occurring in yeast rather than to the fact that the vitamin supplement lacked members of the B complex. In these experiments the increased weight of the liver is used as an index of the deposition of excess fat. Comparison of groups 7 to 11, table 2, demonstrates that the rats which survived hemorrhagic degeneration during the 10-day period grew nearly as well on the injurious AC 32 ration during a subsequent 20-day period as normal rats on the stock diet (group 11). The liver weight remained excessive in groups 8 and 9 but decreased in group 10 which was not continued on the low choline regime. The marked increase in kidney weight occurring during the hemorrhagic phase was no longer evident in the 30-day animals. Although all rats on diet AC 32 showed renal hemorrhage after 10 days, in 50% of a similar group the kidneys appeared normal after 30 days (groups 3 and 7). Such extensive renal repair did not occur in rats suffering the more severe effect of diet AC 39 in the first 10 days (groups 8 and 9). The kidneys of most of these animals showed the characteristic discoloration, mottling or scarring (without hemorrhage). Similar unpublished results have been obtained in rats fed the stock diet for 15 months following the preliminary 10-day period on diet AC 39.

Although the experiments in table 2 indicate that rats which survive acute renal degeneration may continue to grow in spite of the persisting renal damage, the continuation of growth depends upon the diet and upon the severity of the original renal lesions. This is illustrated by the results obtained following the inclusion of cholesterol in diets AC 51 and CG 3 (table 3). Groups 1 to 5 show the opposing effects of choline and cholesterol and the protective action of choline in diet AC 51. The average daily food intakes of the rats in groups 1 to 5 were 4.2, 5.1, 5.3, 5.5 and 5.4 gm. respectively. Groups 6 to 16 were fed CG 3, the most severe diet which has

been used in this study. The average daily food intakes of groups 6 to 9 were 4.9, 5.1, 5.1 and 3.8 gm. respectively. It is of significance that the food consumption was not affected until after the appearance of the renal lesions. The liver fat was excessive on the second day, renal hemorrhage occurred after 3 days and 25% of the animals were dead by the eighth day (groups 6 to 9). These remarkable effects, which were prevented by choline (groups 10 and 11), were intensified by the presence of cholesterol in a diet in which the labile methyl supply had been diminished by decreasing the level of the high-methionine protein, casein. The damaging effects of this diet continued to appear during the so-called recovery phase. Only nine rats out of twenty-seven (group 16) survived for 75 days, the highest mortality occurring between the sixth and ninth and between the thirty-fifth and forty-fifth days. In the nine surviving animals, the livers were exceptionally fatty and the kidneys were grossly and microscopically abnormal. One rat showed evidence of recent renal hemorrhage.

The results with diet CG 3 were also unusual because for the first time in our experiments, choline failed to afford complete protection. This failure was not at all evident during the crucial 8-day period but appeared during a subsequent 22-day period. Groups 12 to 15, table 3, showed an increasing level of chloroform soluble substances in the liver and in four of the eleven rats in group 15 renal hemorrhage, as well as ocular hemorrhage, occurred. These later effects appearing in rats on diets containing cholesterol and choline are being investigated further.

Effect of food consumption on hemorrhagic degeneration. Table 4 (groups 1 to 3) shows the effect of a diet (AA) in which protein was replaced by an amino-acid mixture lacking the sulphur-containing acids. The addition of cystine increased the incidence of renal lesions and these were prevented by methionine. The moderate injury occurring in group 1 seemed unusual in view of the fact that this food mixture was very deficient in supplies of labile methyl. Diet AA was an unsatisfactory ration, possibly because of impalatability and

TABLE 3

The effect of cholesterol on severity of hemorrhagic degeneration in 40-gm. male rats

GROUP [1]	EXPERIMENTAL PERIOD	CHOLINE CHLORIDE ADDED PER GRAM OF FOOD	NO. OF RATS [2]	FINAL BODY WEIGHT	KIDNEY WEIGHT AS PER CENT OF BODY WEIGHT	LIVER FAT AS PER CENT OF LIVER	RENAL LESIONS
	days	*mg.*		*gm.*			%
1	8	0	21–18	51	2.10	21.5	95
2	8	0.35	21–20	63	1.42	20.5	62
3	8	0.5	21–21	66	1.18	17.7	38
4 [3]	8	0.5	21–21	69	1.05	13.0	0
5	8	1.0	21–21	66	1.08	8.8	0
6	2	0	16–16	42	1.29	16.8	0
7	3	0	15–15	47	1.27	18.4	7
8	4	0	14–14	49	1.36	26.7	71
9	8	0	24–18	47	2.38	26.9	100
10	8	4	21–21	62	1.14	5.5	0
11	8	6	14–14	65	1.04	4.9	0
12	15	4	18–18	97	0.98	6.1	0
13	25	4	22–22	116	1.03	8.6	0
14	25	6	24–24	122	0.99	8.6	0
15	40	4	11–11	176	0.86	9.8	36
16	75	0	27– 9	190	0.92	34.9	100

[1] Groups 1 to 5, Basal AC 51 + 1% cholesterol. Groups 6 to 16, Basal CG 3 (0.5% cholesterol).

[2] Second figure shows the number of surviving rats.

[3] Cholesterol omitted.

lack of sulphur amino-acids, and the rats in group 1 consumed too little food for even moderate growth. This failure of appetite was evident from the first day of the experimental period. The average food intakes for the first 4 and the last 3 days were 2.1 and 2.0 gm. respectively. The corresponding food intakes of group 4 (diet AC 39) were 3.6 and 1.9 gm. This diet which differed from AA in the use of casein instead of the amino-acid mixture was consumed in larger amounts at the start of the experiment so that there was some increase in body weight even though the later food intake was no more than that found in group 1. These results suggested the possibility that a deficiency of choline or of labile methyl would result in hemorrhagic degeneration only if the metabolic demands of the maintenance and growth processes were great enough to require more labile methyl than was supplied in

TABLE 4

The effect of food intake and of various antihemorrhagic factors on occurrence of hemorrhagic degeneration in 20-day-old male rats during a 7-day experimental period

GROUP	DIET	NO. OF RATS	AVERAGE BODY WEIGHT		AVERAGE FOOD PER DAY	WEIGHT AS PER CENT OF BODY WEIGHT		RENAL LESIONS
			Initial	Final		Kidneys	Liver	
			gm.	*gm.*	*gm.*			%
1	AA	8	30	29	2.1	1.40	5.5	13
2	AA + 0.3% cystine	16	30	30	2.0	1.72	5.4	69
3	AA + 0.3% cystine and 0.6% dl-methionine	20	29	32	2.3	1.46	4.4	0
4	AC 39	10	30	36	2.9	2.37	7.6	100
5	AC 39 + 6% yeast [1]	10	30	47	3.9	1.47	6.4	100
6	AC 39 + 0.3% choline chloride	10	30	44	3.7	1.18	4.1	0
7	AC 39. Food restricted	10	31	34	2.4	1.77	6.8	100
8	AC 39. Food restricted	10	31	32	1.9	1.17	4.6	10
9	AC 32	19	29	40	3.3	1.90	6.8	90
10	AC 32. Food restricted	20	30	35	2.0	1.23	4.9	0
11	AC 32 + vitamin K [2]	12	27	39	..	1.89	8.3	92
12	AC 32 + vitamin K [2]	10	27	38	..	2.04	7.0	90
13	AC 32 + ascorbic acid [4]	21	29	41	3.8	2.0	7.4	93
14	AC 32 + vitamin P [5]	14	28	41	3.9	1.79	7.5	93
15	AC 32 + vitamin P [6]	7	30	45	3.6	1.70	7.3	100
16	AC 32 + 0.038% choline chloride	22	30	52	5.3	1.20	6.1	0
17	Stock diet, 8-day period	21	40	68	..	1.21	4.6	0
18	Stock diet, 5.5 days. Fasting, 2.5 days	21	40	42	..	1.37	4.0	0

[1] 6% yeast in place of vitamin supplement.

[2] Oral administration of 100 chick units of vitamin K_1 per day in form of an alfalfa extract (oil).

[3] Subcutaneous injection of 400 chick units of the potent oil per day for first 4 days. Daily intraperitoneal injection of 0.3 mg. of 4-amino-2 methyl-1-naphthol hydrochloride.

[4] Oral administration of 1 mg. of ascorbic acid per day. Also 1 mg. added per gram of food.

[5] 5 mg. of "hesperidin" per gram of food.

[6] 20 mg. of "lemon citrin" per gram of food.

the ration. The definite relation between the occurrence of renal lesions, the level of ingested food and the rate of growth was demonstrated by the absence of renal pathology in rats fed restricted amounts of diets which were highly injurious if fed ad libitum. Renal lesions were severe and the liver weight was markedly increased on diet AC 39 (group 4) even if the incomplete vitamin supplement was replaced by yeast (group 5). These effects also occurred if the food intake was restricted to 2.4 gm. per day but not if the food was limited to 1.9 gm. per day (groups 7 and 8). Similarly, restriction of group 10 to 2.0 gm. of diet AC 32 per day resulted in complete protection whereas the unlimited consumption of the same diet produced the characteristic renal damage and increase in liver weight (group 9). Fasting for 60 hours was without evident effect upon liver and kidney weights, expressed as per cent of body weight (groups 17 and 18).

Effect of antihemorrhagic dietary factors on hemorrhagic degeneration. The occurrence of renal and ocular hemorrhage in rats on a low choline, low labile methyl diet made it desirable to determine the possible effect of other antihemorrhagic factors. The results in table 4, groups 11 to 15, indicated that hemorrhagic degeneration was unaffected by vitamin K, by ascorbic acid and by material supplying a possible "vitamin P".

Effect of thiamine chloride, riboflavin, pyridoxine, calcium pantothenate and nicotinic acid on hemorrhagic degeneration. The influence of these members of the vitamin B complex was determined by noting the effect of supplements of each in diet AC 50, alone and with partially protective levels of choline (table 5). Basal diet AC 50 contained 6% of yeast so that in these experiments the individual vitamins were added to a diet already adequate, except in choline. The ingestion of extra thiamine, riboflavin, pyridoxine and calcium pantothenate was without significant effect upon the severity of the choline deficiency. However, the two levels of choline used in these experiments were not as protective in the diets containing nicotinic acid as in the control diets. The sensitivity of

TABLE 5

The effect of vitamins of the B-complex on severity of hemorrhagic degeneration in male rats, 40 gm. in weight and 21 to 26 days of age, during an 8-day experimental period

DIET [1]	NO. OF RATS	FINAL BODY WEIGHT	AVERAGE FOOD INTAKE PER DAY	KIDNEY WEIGHT AS PER CENT OF BODY WEIGHT	LIVER FAT AS PER CENT OF LIVER	RENAL LESIONS
		gm.	*gm.*			%
Basal	42	50	4.3	2.01	18.0	93
+ Thiamine chloride	42	53	4.4	1.91	21.6	86
+ Riboflavin	43	53	4.5	1.97	20.5	98
+ Pyridoxine	21	55	4.6	1.65	21.2	81
+ Calcium pantothenate	42	51	4.3	2.02	21.1	98
+ Nicotinic acid	43	49	4.4	2.20	22.3	95
Basal with 0.02% choline chloride	42	59	5.0	1.28	19.9	52
+ Thiamine chloride	42	62	5.1	1.28	19.7	69
+ Riboflavin	42	60	5.1	1.41	19.8	64
+ Pyridoxine	42	61	5.2	1.39	19.0	79
+ Calcium pantothenate	42	60	5.0	1.29	16.9	62
+ Nicotinic acid	21	56	4.8	1.61	19.9	90
Basal with 0.04% choline chloride	21	63	5.3	1.09	12.5	10
+ Thiamine chloride	21	65	5.4	1.03	12.5	10
+ Riboflavin	21	66	5.5	1.00	12.8	5
+ Pyridoxine	21	67	5.7	0.96	12.6	0
+ Nicotinic acid	21	60	5.2	1.05	19.9	14

[1] Basal diet, AC 50. Indicated vitamin supplements were at the following levels: nicotinic acid, 0.5 mg. per gram of food; all others, 0.05 mg. per gram of food.

these animals to variations in the labile methyl supply suggests a diversion of a part of this supply to the formation of trigonelline as an explanation of the apparent choline-opposing action of nicotinic acid (Ackermann, '12).

DISCUSSION

Hemorrhagic degeneration occurs on certain low choline, low labile methyl diets fed ad libitum but is prevented if the food intake is restricted. This observation is of considerable importance because the low food intake of rats on diets with multiple deficiencies may mask the labile methyl deficiency. Improvement of food consumption, even though slight and

temporary, would stimulate metabolism and thus increase the effect of the methyl deficiency so that hemorrhagic degeneration might occur. Such an effect would not necessarily indicate a direct antagonistic relation between choline or the labile methyl supply and the agent causing the increase in the food intake.

This demonstration of the influence of the food intake and of growth on the effects of choline deficiency confirms the earlier statements that "the deposition of liver fat was intensified on those diets permitting the better rates of growth" (Griffith and Wade, '39), and that "the renal lesions were produced more readily if the dietary protein was adequate in amount and in composition for good growth" (Griffith and Wade, '40). In view of our failure to demonstrate an antagonistic relation between thiamine or pyridoxine and choline as reported by McHenry ('37) and by Gyorgy and Goldblatt ('40), respectively, it is suggested that their results may be explained on the basis of an indirect effect of these two vitamins on the food intake or on the rate of growth of the experimental rats. In one experiment, McHenry equalized the food consumption of two groups of rats on a low choline basal diet, with and without supplementary thiamine, and found a greater deposition of liver fat in the thiamine-fed group. These animals, however, grew at a faster rate than the controls fed the same amount of the basal diet and may have used more of the labile methyl supply for the synthesis of methylated compounds other than choline.

The experiments of Griffith and Wade ('39 and '40) demonstrated the importance of choline and of methionine in the maintenance of the normal renal structure in young rats. At the same time, du Vigneaud showed that the methyls of choline (du Vigneaud, Chandler, Moyer and Keppel, '39) and of betaine (Chandler and du Vigneaud, '40) may be transferred to homocysteine and suggested that "the presence of methyl groups in a utilizable form, such as, methionine or choline, may be essential in the diet" (du Vigneaud, Chandler, Cohn and Brown, '40). Our own observations support such a con-

clusion and, in addition, point to hemorrhagic degeneration as the specific effect of a dietary deficiency of the labile methyl supply. The various manifestations of hemorrhagic degeneration may be due to the failure of synthesis in the body of one or more of the many methylated compounds important as constituents of tissues, as metabolites or as detoxication products of toxic substances.

Choline is of prime importance as a lipotropic substance and as a source of labile methyl. In this connection it may be of unusual significance that dietary supplements of cholesterol produce injurious effects which appear after 30 to 40 days even though the diet contains more than the amount of choline required for complete protection of the rat during the crucial 8-day period. Possibly, the continued prevention of the toxic effect of cholesterol is dependent upon the presence in the food mixture of choline and of some other indispensable compound, the lack of which becomes evident only in longer experimental periods.

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SUMMARY

1. A dietary deficiency of choline and of the labile methyl supply in young rats produces a marked elevation of the nonprotein nitrogen of the blood coincident with renal hemorrhagic degeneration.

2. Survival, renal repair and resumption of growth depend upon the severity of the acute phase.

3. The aggravating effect of dietary cholesterol is prevented by choline during the crucial 8-day period but not during a subsequent 30-day period.

4. Supplements of thiamine, riboflavin, pyridoxine, calcium pantothenate, ascorbic acid, vitamin K and "vitamin P" do not affect the severity of the results of labile methyl deficiency whereas nicotinic acid exerts a moderate choline-opposing action.

5. Hemorrhagic degeneration, which is severe if diets are fed ad libitum, may be prevented by partial restriction of the consumption of food.

6. The deposition of liver fat or the appearance of renal hemorrhage in experiments in which a dietary supplement increases the consumption of food or the rate of growth is not necessarily evidence of a direct antagonism between choline and the dietary supplement.

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INDEX

ABSORPTION of various fatty acids by the rat, rate of 373
Acid, pantothenic. Quantitative study of vitamins in the rumen content of sheep and cows fed vitamin-low diets. IV 405
Acid, pantothenic, content of eggs, effect of diet on 201
Acid, pantothenic, distribution of, in certain products of natural origin 193
Acid, pantothenic, requirement of mouse for, and for new factor of vitamin B complex 609
Acid, phytic, yeast nucleic acid, soybean phosphatides and inorganic salts as sources of phosphorus for bone calcification 213
Acid, yeast nucleic, phytic acid, soybean phosphatides and inorganic salts as sources of phosphorus for bone calcification 213
Acids, amino, specific dynamic effects of, and their bearing on the causes of specific dynamic effects of proteins 257
Acids, organic, and salts, certain, with particular reference to their effect on ossification, biologic response of chickens to 515
Acids, various fatty, rate of absorption of, by the rat 373
Acquisition of fluorine by mature teeth, on the manner of 35
Action, sparing, of thiamine on body tissue catabolism 445
Administration of l(—)histidine to the dog, heat production and blood and urine constituents after 25
Adult man, further experiments on the calcium requirement of, and the utilization of the calcium in milk 577
Adults, seven, balance studies on. Calcium requirement of man 565
Adults, utilization of the calcium of milk by 351
Age, given, calcium content of the normal growing body at 155
ALINE, DONNA E. See Snell, Esmond E. 201
ALMQUIST, HERMAN J. Chemical estimation of quality in animal protein concentrates 347
Amino acids, specific dynamic effects of, and their bearing on the causes of specific dynamic effects of proteins 257
Animal protein concentrates, chemical estimation of quality in 347
ANSBACHER, STEFAN. The bioassay of vitamin K 1
Anticataractogenic quality of certain proteins, effect of dry heat upon 115
ARMSTRONG, WALLACE D. See Perry, Mabel W. 35
ARMSTRONG, WILLIAMINA. See Breiter, Herta 351
ARMSTRONG, WILLIAMINA. See Outhouse, Julia 565
Arsenic and goiter 341
A.T.10 (dihydrotachysterol), effect of, on rickets in rats produced by high-calcium-low-phosphorus diets 147
Avitaminosis, B_1, analogous to Wernicke's disease of man. A deficiency disease of foxes produced by feeding fish 243

B_1 avitaminosis analogous to Wernicke's disease of man. A deficiency disease of foxes produced by feeding fish 243
BACHMANN, GEORGE. See Haldi, John 307
Balance studies on seven adults. Calcium requirement of man 565
Balances, iron, on four normal preschool children 101
Basal food intake, effect of quantity of, upon the utilization of vitamin A 13
Basal metabolism and heat loss of young women at temperatures from 22°C. to 35°C. Clinical calorimetry no. 54 383
BAUMANN, CARL A., ELTON G. FOSTER AND PAUL S. LAVIK. The effect of certain carcinogens on vitamin A in the liver 431
BETHKE, ROLAND M. See Hunt, Charles H. 85
Bioassay of vitamin K 1
Biologic response of chickens to certain organic acids and salts with particular reference to their effect on ossification 515
BLATHERWICK, NORMAN R. See Rynbergen, Henderika J. 553
Blood and urine constituents, and heat production, after administration of l(—)histidine to the dog 25
Blood plasma, cattle, carotene and vitamin A in, with observations on reproductive performance at restricted levels of carotene intake .. 135
BODANSKY, MEYER, AND VIRGINIA B. DUFF. Effects of parathyroid deficiency and calcium and phosphorus of the diet on pregnant rats 179
BODANSKY, MEYER, AND VIRGINIA B. DUFF. Relation of parathyroid function and diet to the mineral composition of the bones in rats at the conclusion of pregnancy 235
Body, normal growing, at a given age, calcium content of 155
Body tissue catabolism, sparing action of thiamine on 445
BOELTER, MURIEL D. D., AND DAVID M. GREENBERG. Severe calcium deficiency in growing rats. I. Symptoms and pathology 61
BOELTER, MURIEL D. D., AND DAVID M. GREENBERG. Severe calcium deficiency in growing rats. II. Changes in chemical composition . 75

BOELTER, MURIEL D. D. See Kleiber, Max 363
Bone calcification, phytic acid, yeast nucleic acid, soybean phosphatides and inorganic salts as sources of phosphorus for 213
Bones in rats at conclusion of pregnancy, relation of parathyroid function and diet to mineral composition of 235
BREITER, HERTA, ROSALIND MILLS, JULIA DWIGHT, BEULA MCKEY, WILLIAMINA ARMSTRONG AND JULIA OUTHOUSE. The utilization of the calcium of milk by adults 351
BREITER, HERTA. See Outhouse, Julia 565
BRIWA, KATHRYN E., AND HENRY C. SHERMAN. The calcium content of the normal growing body at a given age 155
BUEL, FLORENCE. See Strouse, Solomon 599
BUNKFELDT, RUDOLF. See Krieger, Carl H. 213
BURROUGHS, EDWARD W. See Hunt, Charles H. 85

CAFFEINE, effect of various amounts of, on the gaseous exchange and the respiratory quotient in man 307
CAHILL, WILLIAM M. Urinary excretion of thiamine on high fat and high carbohydrate diets 411
Calcification, bone, phytic acid, yeast nucleic acid, soybean phosphatides and inorganic salts as sources of phosphorus for 213
Calcium and phosphorus, effect of, on metabolism of lead 541
Calcium and phosphorus of the diet, effects of parathyroid deficiency and, on pregnant rats 179
Calcium content of the normal growing body at a given age 155
Calcium deficiency, severe, in growing rats. I. Symptoms and pathology 61
Calcium deficiency, severe, in growing rats. II. Changes in chemical composition 75
Calcium (high)-low-phosphorus diets, effect of AT.10 (dihydrotachysterol) on rickets in rats produced by 147
Calcium in milk, utilization of, further experiments on the calcium requirement of adult man and ... 577
Calcium intake, optimal, life-time experiments upon the problem of 221
Calcium of milk, utilization of, by adults 351
Calcium requirement of adult man and the utilization of the calcium in milk, further experiments on ... 577
Calcium requirement of man: Balance studies on seven adults 565
Calorimetry, clinical, no. 54. Basal metabolism and heat loss of young women at temperatures from 22°C. to 35°C. 383
Carbohydrate, high, and high fat diets, urinary excretion of thiamine on 411
Carcinogens, effect of certain, on vitamin A in liver 431
CARLSON, WALTER E. See Greene, Robert G. 243
Carotene and vitamin A in cattle blood plasma with observations on reproductive performance at restricted levels of carotene intake 135
Casein and deaminized casein, effect of hydrolytic products of, on the cataractogenic action of galactose 125
Casein, lactalbumin, fibrin, edestin and gelatin in hemorrhagic degeneration in young rats, effect of supplementary choline, methionine and cystine and of 291
Catabolism, body tissue, sparing action of thiamine on 445
Catabolism, fasting, and food utilization of magnesium deficient rats 363
Cataractogenic action of galactose, effect of hydrolytic products of casein and deaminized casein on 125
Cattle blood plasma, carotene and vitamin A in, with observations on reproductive performance at restricted levels of carotene intake 135
Cattle, studies on riboflavin and thiamin in the rumen content of 85
Causes of specific dynamic effects of proteins, specific dynamic effects of amino acids and their bearing on 257
Cereals and rickets. XIII. Phytic acid, yeast nucleic acid, soybean phosphatides and inorganic salts as sources of phosphorus for bone calcification 213
CERECKDO, LEOPOLD R. See Sandza, Joseph G. 609
CHAMBERS, WILLIAM H. See Rynbergen, Henderika J. 553
Chemical composition, changes in. Severe calcium deficiency in growing rats. II 75
Chemical estimation of quality in animal protein concentrates 347
Chickens, biologic response of, to certain organic acids and salts with particular reference to their effect on ossification 515
Children, four normal pre-school, iron balances on 101
Children, four preschool, studies on vitamin C metabolism of 503
Children (school), group of, study of vitamin C nutrition in. Part II. Dietary evaluation 527
Choline metabolism. V. The effect of supplementary choline, methionine and cystine and of casein, lactalbumin, fibrin, edestin and gelatin in hemorrhagic degeneration in young rats 291
Choline metabolism. VII. Some dietary factors affecting the incidence and severity of hemorrhagic degeneration in young rats 617
Choline, methionine and cystine, effect of supplementary, and of casein, lactalbumin, fibrin, edestin and gelatin in hemorrhagic degeneration in young rats 291
CITRON, MARTIN M. See Sure, Barnett 453
Clinical calorimetry no. 54. Basal metabolism and heat loss of young women at temperatures from 22°C. to 35°C. 383
Cobalt in dogs on milk diets, a study of the need for 93
Cod liver oil, uncomplicated vitamin E deficiency in the rabbit and its relation to the toxicity of 225
COLOVOS, NICHOLAS F. See Lee, Robert C. 321
Complex, vitamin B, new factor of, requirement of mouse for pantothenic acid and for 609

Composition, chemical, changes in. Severe calcium deficiency in growing rats. II 75
Composition, mineral, of bones in rats at conclusion of pregnancy, relation of parathyroid function and diet to 235
Concentrates, animal protein, chemical estimation of quality in 347
CORRELL, JOHN T. The biologic response of chickens to certain organic acids and salts with particular reference to their effect on ossification 515
COUCH, JAMES R. See Snell, Esmond E. 201
COWGILL, GEORGE R. See Street, Harold R. 275
Cows, and sheep, fed vitamin-low diets, quantitative study of vitamins in the rumen contents of. III. Thiamin 163
Cows, and sheep, fed vitamin-low diets, quantitative study of vitamins in the rumen content of. IV. Pantothenic acid 405
Cystine, choline and methionine, effect of supplementary, and of casein, lactalbumin, fibrin, edestin and gelatin in hemorrhagic degeneration in young rats 291

DAVIS, RUSSELL E., AND LOUIS L. MADSEN. Carotene and vitamin A in cattle blood plasma with observations on reproductive performance at restricted levels of carotene intake 135
Deaminized casein, and casein, effect of hydrolytic products of, on the cataractogenic action of galactose 125
Deficiency disease of foxes produced by feeding fish. B_1 avitaminosis analogous to Wernicke's disease of man 243
Deficiency, parathyroid, and calcium and phosphorus of the diet on pregnant rats, effects of 179
Deficiency, severe calcium, in growing rats. I. Symptoms and pathology 61
Deficiency, severe calcium, in growing rats. II. Changes in chemical composition 75
Deficiency, uncomplicated vitamin E, in the rabbit and its relation to the toxicity of cod liver oil 225
Deficiency, vitamin A: A field study in Newfoundland and Labrador .. 461
Deficiency, vitamin B_6, in the dog, some observations of 275
Deficiency, vitamin, perosis due to ... 327
Deficient (magnesium) rats, fasting catabolism and food utilization of 363
Degeneration, hemorrhagic, in young rats, effect of supplementary choline, methionine and cystine and of casein, lactalbumin, fibrin, edestin and gelatin in 291
Degeneration, hemorrhagic, in young rats, some dietary factors affecting incidence and severity of 633
Depletion of tissue glycogen during fasting and fatigue and partial recovery without food 477
DEUEL, HARRY J., JR., LOIS HALLMAN AARON REIFMAN. The rate of absorption of various fatty acids by the rat 373
Diabetes, experimental, and diabetes mellitus, sodium and potassium salts in the treatment of 599
Diabetes mellitus, and experimental diabetes, sodium and potassium salts in the treatment of 599
DICHEK, MAURICE. See Sure, Barnett 445, 453
Diet, and parathyroid function, relation of, to mineral composition of bones in rats at conclusion of pregnancy 235
Diet, effect of, on the pantothenic acid content of eggs 201
Diet, effects of parathyroid deficiency and calcium and phosphorus of, on pregnant rats 179
Dietary evaluation (part II). A study of vitamin C nutrition in a group of school children 527
Dietary factors, some, affecting incidence and severity of hemorrhagic degeneration in young rats 633
Diets, high-calcium-low-phosphorus, effect of A.T.10 (dihydrotachysterol) on rickets in rats produced by 147
Diets, high fat and high carbohydrate, urinary excretion of thiamine on 411
Diets, milk, a study of the need for cobalt in dogs on 93
Diets, vitamin-low, quantitative study of vitamins in the rumen content of sheep and cows fed. III. Thiamin 163
Diets, vitamin-low, quantitative study of vitamins in the rumen content of sheep and cows fed. IV. Pantothenic acid 405
Dihydrotachysterol (A.T.10), effect of, on rickets in rats produced by high-calcium-low-phosphorus diets 147
Disease, deficiency, of foxes produced by feeding fish. B_1 avitaminosis analogous to Wernicke's disease of man 243
Disease, Wernicke's, of man, B_1 avitaminosis analogous to. A deficiency disease of foxes produced by feeding fish 243
Distribution of pantothenic acid in certain products of natural origin 193
Distribution of pyridoxine (vitamin B_6) in meat and meat products .. 589
Dog, heat production and blood and urine constituents after administration of l(—)histidine to 25
Dog, some observations of vitamin B_6 deficiency in 275
Dogs on milk diets, a study of the need for cobalt in 93
DOTY, JOHN R. See Eaton, Alonzo G. 25
DRURY, DOUGLAS. See Strouse, Solomon 599
Dry heat, effect of, upon anticataractogenic quality of certain proteins 115
DU BOIS, EUGENE F. See Hardy, James D. 383
DUFF, VIRGINIA B. See Bodansky, Meyer 179, 235
DWIGHT, JULIA. See Breiter, Herta 351
DWIGHT, JULIA. See Outhouse, Julia 565
Dynamic effects, specific, of amino acids and their bearing on the causes of specific dynamic effects of proteins 257

EATON, ALONZO G., AND JOHN R. DOTY. The heat production and blood and urine constituents after administration of l(—)histidine to the dog 25
Economy of food utilization, riboflavin as a factor in 453

Edestin, casein, lactalbumin, fibrin and gelatin in hemorrhagic degeneration in young rats, effect of supplementary choline, methionine and cystine and of 291
Editorial Review. Bioassay of vitamin K 1
Effect of A.T.10 (dihydrotachysterol) on rickets in rats produced by high-calcium-low-phosphorus diets 147
Effect of calcium and phosphorus on the metabolism of lead 541
Effect of certain carcinogens on vitamin A in the liver 431
Effect of diet on the pantothenic acid content of eggs 201
Effect of dry heat upon the anticataractogenic quality of certain proteins 115
Effect of hydrolytic products of casein and deaminized casein on the cataractogenic action of galactose 125
Effect of quantity of basal food intake upon utilization of vitamin A .. 13
Effect of supplementary choline, methionine and cystine and of casein, lactalbumin, fibrin, edestin and gelatin in hemorrhagic degeneration in young rats 291
Effect of various amounts of caffein on the gaseous exchange and the respiratory quotient in man 307
Effect on ossification, biologic response of chickens to certain organic acids and salts with particular reference to their 515
Effects of parathyroid deficiency and calcium and phosphorus of the diet on pregnant rats 179
Effects, specific dynamic, of amino acids and their bearing on the causes of specific dynamic effects of proteins 257
Eggs, effect of diet on pantothenic acid content of 201
ELVEHJEM, CONRAD A. See Frost, Douglas Van Anden 93
ELVEHJEM, CONRAD A. See Henderson, LaVell M. 589
ENSOR, CHARLES. See Haldi, John ... 307
Estimation, chemical, of quality in animal protein concentrates 347
EVANS, CHARLES A. See Green, Robert G. 243
Excretion, urinary, of thiamine on high fat and high carbohydrate diets 411
Exhaustion, extreme muscular, glycogen formation in liver and muscle from glucose and fructose after 489
Experimental diabetes and diabetes mellitus, sodium and potassium salts in the treatment of 599
Experiments, further, on the calcium requirement of adult man and the utilization of the calcium in milk 577

FACTOR in economy of food utilization, riboflavin as 453
Factor, new, of vitamin B complex, requirement of mouse for pantothenic acid and for 609
Factors, some dietary, affecting incidence and severity of hemorrhagic degeneration in young rats 633
FARBER, SIDNEY. See Shohl, Alfred T. 147
Fasting and fatigue and partial recovery without food, depletion of tissue glycogen during 477
Fasting catabolism and food utilization of magnesium deficient rats ... 363
Fat, high, and high carbohydrate diets, urinary excretion of thiamine on 411
Fatigue and fasting, and partial recovery without food, depletion of tissue glycogen during 477
Fattening, and growth, of swine, optimum level of protein intake for 419
Fatty acids, various, rate of absorption of, by the rat 373
Fibrin, casein, lactalbumin, edestin and gelatin in hemorrhagic degeneration in young rats, effect of supplementary choline, methionine and cystine and of 291
Field study in Newfoundland and Labrador. Vitamin A deficiency 461
FINKELSTEIN, BEATRICE. See Lanford, Caroline S. 175
Fish, deficiency disease of foxes produced by feeding B_1 avitaminosis analogous to Wernicke's disease of man 243
Flourine, on the manner of acquisition of, by mature teeth 35
Food, depletion of tissue glycogen during fasting and fatigue and partial recovery without 477
Food intake, basal, effect of quantity of, upon the utilization of vitamin A 13
Food utilization, and fasting catabolism, of magnesium deficient rats 363
Food utilization, riboflavin as a factor in economy of 453
FOSTER, ELTON G. See Baumann, Carl A. 431
Foxes, deficiency disease of, produced by feeding fish. B_1 avitaminosis analogous to Wernicke's disease of man 243
FROST, DOUGLAS VAN ANDEN, CONRAD A. ELVEHJEM AND EDWIN B. HART. A study of the need for cobalt in dogs on milk diets 93
Fructose and glucose, glycogen formation in liver and muscle from, after extreme muscular exhaustion 489
Fructosuria, respiratory metabolism in 553
Fruits, some typical, riboflavin contents of 175
Further experiments on the calcium requirement of adult man and the utilization of the calcium in milk 577

GALACTOSE, effect of hydrolytic products of casein and deaminized casein on the cataractogenic action of 125
Gaseous exchange and respiratory quotient in man, effect of various amounts of caffeine on 307
Gelatin, casein, lactalbumin, fibrin and edestin in hemorrhagic degeneration in young rats, effect of supplementary choline, methionine and cystine and of 291
GERLAUGH, PAUL. See Hunt, Charles H. 85
Glucose and fructose, glycogen formation in liver and muscle from, after extreme muscular exhaustion 489
Glycogen formation in liver and muscle from glucose and fructose after extreme muscular exhaustion ... 489
Glycogen, tissue, depletion of, during fasting and fatigue and partial recovery without food 477

Goat, pig and sheep, skin temperatures of, under winter conditions 321
Goiter, and arsenic 341
Goss, Harold. See McElroy, Laird W. 163, 405
Green, Robert G., Walter E. Carlson and Charles A. Evans. A deficiency disease of foxes produced by feeding fish. B_1 avitaminosis analogous to Wernicke's disease of man 243
Greenberg, David M. See Boelter, Muriel D. D 61, 75
Greenberg, David M. See Kleiber, Max 363
Griffith, Wendell H. Choline metabolism. V. The effect of supplementary choline, methionine and cystine and of casein, lactalbumin, fibrin, edestin and gelatin in hemorrhagic degeneration in young rats 291
Griffith, Wendell H., and Dwight J. Mulford. Choline metabolism. VII. Some dietary factors affecting the incidence and severity of hemorrhagic degeneration in young rats 633
Growing, normal, body at a given age, calcium content of 155
Growing rats, severe calcium deficiency in. I. Symptoms and pathology .. 61
Growing rats, severe calcium deficiency in. II. Changes in chemical composition 75
Growth and fattening of swine, optimum level of protein intake for 419

Haldi, John, George Bachmann, Charles Ensor and Winfrey Wynn. The effect of various amounts of caffeine on the gaseous exchange and the respiratory quotient in man 307
Hallman, Lois. See Deuel, Harry J., Jr. 373
Hardy, James D., Ade T. Milhorat and Eugene F. Du Bois, with the technical assistance of Gustave F. Soderstrom. Basal metabolism and heat loss of young women at temperatures from 22°C. to 35°C. Clinical calorimetry no 54 383
Hart, Edwin B. See Frost, Douglas Van Anden 93
Hathaway, Milicent L., and Frieda L. Meyer. Studies on the vitamin C metabolism of four preschool children 503
Heat, dry, effect of, upon anticataractogenic quality of certain proteins 115
Heat loss, and basal metabolism, of young women at temperatures from 22°C. to 35°C. Clinical calorimetry no. 54 383
Heat production and blood and urine constituents after administration of l(—)histidine to the dog 25
Hemorrhagic degeneration in young rats, effect of supplementary choline, methionine and cystine and of casein, lactalbumin, fibrin, edestin and gelatin in 291
Hemorrhagic degeneration in young rats, some dietary factors affecting incidence and severity of 633
Henderson, LaVell M., Harry A. Waisman and C. A. Elvehjem. The distribution of pyridoxine (vitamin B_6) in meat and meat products 589
Henderson, Mary D., and Helen S. Mitchell. The effect of dry heat upon the antcataractogenic quality of certain proteins 115
Henderson, Mary D. See Moore, Edwin L. 125
High-calcium-low-phosphorus diets, effect of A.T.10 (dihydrotachysterol) on rickets in rats produced by ... 147
High carbohydrate, and high fat diets, urinary excretion of thiamine on 411
High fat and high carbohydrate diets, urinary excretion of thiamine on 411
Histidine, l(—), to the dog, heat production and blood and urine constituents after administration of 25
Hogan, Albert G., Luther R. Richardson, Homer Patrick and Harry L. Kempster. Perosis due to a vitamin deficiency 327
Human subjects, iron metabolism in, on daily intakes of less than 5 milligrams 617
Hunt, Charles H., C. H. Kick, Edward W. Burroughs, Roland M. Bethke, A. F. Schalk and Paul Gerlaugh. Studies on riboflavin and thiamin in the rumen content of cattle 85
Hydrolytic products of casein and deaminized casein, effect of, on cataractogenic action of galactose 125

Incidence and severity of hemorrhagic degeneration in young rats, some dietary factors affecting ... 633
Inorganic salts, phytic acid, yeast nucleic acid and soybean phosphatides as sources of phosphorus for bone calcification 213
Intake, basal food, effect of quantity of, upon the utilization of vitamin A 13
Intake, carotene, carotene and vitamin A in cattle blood plasma with observations on reproductive performance at restricted levels of .. 135
Intake, daily, of less than 5 milligrams, iron metabolism in human subjects on 617
Intake, optimal calcium, life-time experiments upon the problem of 221
Intake, protein, for the growth and fattening of swine, optimum level of 419
Iron balances on four normal preschool children 101
Iron metabolism in human subjects on daily intakes of less than 5 milligrams 617

Jukes, Thomas H. The distribution of pantothenic acid in certain products of natural origin 193

Kay, Ramond. See Strouse, Solomon 599
Keith, Thomas B. See Miller, Russell C. 419
Kelly, Eunice. See Muelder, Kathleen D. 13
Kempster, Harry L. See Hogan, Albert G. 327
Kick, C. H. See Hunt, Charles H. 85

KLEIBER, MAX, MURIEL D. D. BOELTER AND DAVID M. GREENBERG. Fasting catabolism and food utilization of magnesium deficient rats 363

KRIEGER, CARL H., RUDOLF BUNKFELDT, CHESTER R. THOMPSON AND HARRY STEENBOCK. Cereals and rickets. XIII. Phytic acid, yeast nucleic acid, soybean phosphatides and inorganic salts as sources of phosphorus for bone calcification 213

KRISS, MAX. The specific dynamic effects of amino acids and their bearing on the causes of specific dynamic effects of proteins 257

LABRADOR and Newfoundland, a fie'd study in. Vitamin A deficiency 461

l(—)histidine to the dog, heat production and blood and urine constituents after administration of 25

Lactalbumin, casein, fibrin, edestin and gelatin in hemorrhagic degeneration in young rats, effect of supplementary choline, methionine and cystine and of 291

LANFORD, CAROLINE S., BEATRICE FINKELSTEIN AND HENRY C. SHERMAN. Riboflavin contents of some typical fruits 175

LANFORD, CAROLINE S. See Van Duyne, Frances O. 221

Latter half of life, nutritional requirements during 45

LAVIK, PAUL S. See Baumann, Carl A. 431

Lead, effect of calcium and phosphorus on metabolism of 541

LEE, ROBERT C., NICHOLAS F. COLOVOS AND ERNEST G. RITZMAN. Skin temperatures of the pig, goat, and sheep under winter conditions 321

Level, optimum, of protein intake for the growth and fattening of swine 419

Levels, restricted, of carotene intake, carotene and vitamin A in cattle blood plasma with observations on reproductive performance at 135

LEVERTON, RUTH M. Iron metabolism in human subjects on daily intakes of less than 5 milligrams 617

Life, latter half of, nutritional requirements during 45

Life-time experiments upon the problem of optimal calcium intake 221

Liver and muscle, glycogen formation in, from glucose and fructose after extreme muscular exhaustion 489

Liver, vitamin A in, effect of certain carcinogens on 431

Loss, heat, and basal metabolism, of young women at temperatures from 22°C. to 35°C. Clinical calorimetry no. 54 383

Low-phosphorus-high-calcium diets, effect of A.T.10 (dihydrotachysterol) on rickets in rats produced by 147

Low (vitamin) diets, quantitative study of vitamins in the rumen content of sheep and cows fed. III. Thiamin 163

Low (vitamin) diets, quantitative study of vitamins in the rumen content of sheep and cows fed. IV. Pantothenic acid 405

MCCAY, CLIVE M., LEONARD A. MAYNARD, GLADYS SPERLING AND HARLOW S. OSGOOD. Nutritional requirements during the latter half of life 45

MCCOLLUM, E. V. See Mackenzie, Cosmo G. 225

MCELROY, LAIRD W., AND HAROLD GOSS. A quantitative study of vitamins in the rumen content of sheep and cows fed vitamin-low diets. III. Thiamin 163

MCELROY, LAIRD W., AND HAROLD GOSS. A quantitative study of vitamins in the rumen content of sheep and cows fed vitamin-low diets. IV. Pantothenic acid 405

MCKEY, BEULA. See Breiter, Herta 351

MACKENZIE, COSMO G., JULIA B. MACKENZIE AND E. V. MCCOLLUM. Uncomplicated vitamin E deficiency in the rabbit and its relation to the toxicity of cod liver oil 225

MACKENZIE, JULIA B. See Mackenzie, Cosmo G. 225

MADSEN, LOUIS L. See Davis, Russell E. 135

Magnesium deficient rats, fasting catabolism and food utilization of 363

Man, adult, further experiments on the calcium requirement of, and the utilization of the calcium in milk 577

Man, calcium requirement of. Balance studies on seven adults 565

Man, effect of various amounts of caffeine on the gaseous exchange and the respiratory quotient in 307

Man, Wernicke's disease of, B_1 avitaminosis analogous to. A deficiency disease of foxes produced by feeding fish 243

Manner of acquisition of fluorine by mature teeth, on the 35

Mature teeth, on the manner of acquisition of fluorine by 35

MAYNARD, LEONARD A. See McKay, Clive M. 45

Meat and meat products, distribution of pyridoxine (vitamin B_6) in 589

Metabolism, basal, and heat loss of young women at temperatures from 22°C. to 35°C. Clinical calorimetry no. 54 383

Metabolism, choline. V. The effect of supplementary choline, methionine and cystine and of casein, lactalbumin, fibrin, edestin and gelatin in hemorrhagic degeneration in young rats 291

Metabolism, choline. VII. Some dietary factors affecting the incidence and severity of hemorrhagic degeneration in young rats 633

Metabolism, iron, in human subjects on daily intakes of less than 5 milligrams 617

Metabolism of lead, effect of calcium and phosphorus on 541

Metabolism, respiratory, in fructosuria 553

Metabolism, vitamin C, of four preschool children, studies on 503

Methionine, choline and cystine, effect of supplementary, and of casein, lactalbumin, fibrin, edestin and gelatin in hemorrhagic degeneration in young rats 291

METZGER, MARGARET. See Sharpless, George R. 341

MEYER, FRIEDA L. See Hathaway, Milicent L. 503
MILHORAT, ADE T. See Hardy, James D. 383
Milk, calcium of, utilization of, by adults 351
Milk diets, a study of the need for cobalt in dogs on 93
Milk, utilization of calcium in, further experiments on the calcium requirement of adult man and ... 577
MILLER, RUSSELL C., AND THOMAS B. KEITH. The optimum level of protein intake for the growth and fattening of swine 419
MILLS, ROSALIND. See Breiter, Herta 351
MILLS, ROSALIND. See Outhouse, Julia 565
Mineral composition of bones in rats at conclusion of pregnancy, relation of parathyroid function and diet to 235
MITCHELL, HELEN S. See Henderson, Mary D. 115
MITCHELL, HELEN S. See Moore, Edwin L. 125
MITCHELL, H. H. See Shields, John B. 541
MITCHELL, H. H. See Steggerda, Frederic R. 577
MOORE, EDWIN L., MARY D. HENDERSON, HELEN S. MITCHELL AND WALTER S. RITCHIE. The effect of the hydrolytic products of casein and deaminized casein on the cataractogenic action of galactose 125
Mouse, requirement of, for pantothenic acid and for a new factor of the vitamin B complex 609
MUELDER, KATHLEEN D., AND EUNICE KELLY. The effect of the quantity of basal food intake upon the utilization of vitamin A 13
MULFORD, DWIGHT J. See Griffith, Wendell H. 633
MURLIN, JOHN R. See Nutter, Pauline E. 489
MURPHY, ELIZABETH. A study of vitamin C nutrition in a group of school children. Part II. Dietary evaluation 527
Muscle and liver, glycogen formation in, from glucose and fructose after extreme muscular exhaustion ... 489
Muscular exhaustion, extreme, glycogen formation in liver and muscle from glucose and fructose after 489

NATURAL origin, distribution of pantothenic acid in certain products of 193
Need for cobalt in dogs on milk diets, study of 93
Newfoundland and Labrador, a field study in. Vitamin A deficiency 461
Normal growing body at a given age, calcium content of 155
Normal pre-school children, four, iron balances on 101
Nucleic acid, yeast, phytic acid, soybean phosphatides and inorganic salts as sources of phosphorus for bone calcification 213
Nutrition, vitamin C, in a group of school children, study of. Part II. Dietary evaluation 527
Nutritional requirements during the latter half of life 45
NUTTER, PAULINE E. Depletion of tissue glycogen during fasting and fatigue and partial recovery without food 477
NUTTER, PAULINE E., AND JOHN R. MURLIN. Glycogen formation in liver and muscle from glucose and fructose after extreme muscular exhaustion 489

OBSERVATIONS, some, of vitamin B_6 deficiency in the dog 275
On the manner of acquisition of fluorine by mature teeth 35
Optimal calcium intake, life-time experiments upon the problem of 221
Optimum level of protein intake for the growth and fattening of swine ... 419
Organic acids and salts, certain, with particular reference to their effect on ossification, biologic response of chickens to 515
Origin, natural, distribution of pantothenic acid in certain products of 193
OSGOOD, HARLOW S. See McCay, Clive M. 45
Ossification, effect on, biological response of chickens to certain organic acids and salts, with particular reference to their 515
OUTHOUSE, JULIA, HERTA BREITER, ESTHER RUTHERFORD, JULIA DWIGHT, ROSALIND MILLS AND WILLIAMINA ARMSTRONG. The calcium requirement of man. Balance studies on seven adults .. 565
OUTHOUSE, JULIA. See Breiter, Herta 351

PANTOTHENIC acid content of eggs, effect of diet on 201
Pantothenic acid, distribution of, in certain products of natural origin 193
Pantothenic acid. Quantitative study of vitamins in the rumen content of sheep and cows fed vitamin-low diets. IV. 405
Pantothenic acid, requirement of mouse for, and for new factor of vitamin B complex 609
Parathyroid deficiency and calcium and phosphorus of the diet on pregnant rats, effects of 179
Parathyroid function and diet, relation of, to mineral composition of bones in rats at conclusion of pregnancy 235
Pathology, and symptoms. Severe calcium deficiency in growing rats. I. 61
PATRICK, HOMER. See Hogan, Albert G. 327
PEARSON, PAUL B. See Snell, Esmond E. 201
Perosis due to a vitamin deficiency 327
PERRY, MABEL W., AND WALLACE D. ARMSTRONG. On the manner of acquisition of fluorine by mature teeth 35
Phosphatides, soybean, phytic acid, yeast nucleic acid and inorganic salts as sources of phosphorus for bone calcification 213
Phosphorus and calcium, effect of, on metabolism of lead 541
Phosphorus and calcium of the diet, effects of parathyroid deficiency and, on pregnant rats 179
Phosphorus for bone calcification, phytic acid, yeast nucleic acid, soybean phosphatides and inorganic salts as sources of 213

Phosphorus (low)-high-calcium diets, effect of A.T.10 (dihydrotachysterol) on rickets in rats produced by 147
Pig, goat and sheep, skin temperatures of, under winter conditions 321
Plasma, cattle blood, carotene and vitamin A in, with observations on reproductive performance at restricted levels of carotene intake 135
Phytic acid, yeast nucleic acid, soybean phosphatides and inorganic salts as sources of phosphorus for bone calcification 213
PORTER, THELMA. Iron balances on four normal pre-school children .. 101
Potassium, and sodium, salts in the treatment of experimental diabetes and diabetes mellitus 599
Pregnancy, relation of parathyroid function and diet to mineral composition of bones in rats at conclusion of 235
Pregnant rats, effects of parathyroid deficiency and calcium and phosphorus of the diet on 179
Pre-school children, four normal, iron balances on 101
Preschool children, four, studies on vitamin C metabolism of 503
Protein concentrates, animal, chemical estimation of quality in 347
Protein intake for the growth and fattening of swine, optimum level of 419
Proteins, certain, effect of dry heat upon anticataractogenic quality of 115
Proteins, specific dynamic effects of amino acids and their bearing on the causes of specific dynamic effects of 257
Pyridoxine (vitamin B_6), distribution of, in meat and meat products ... 589

QUALITY in animal protein concentrates, chemical estimation of 347
Quantitative study of vitamins in the rumen content of sheep and cows fed vitamin-low diets. III. Thiamin 163
Quantitative study of vitamins in the rumen content of sheep and cows fed vitamin-low diets. IV. Pantothenic acid 405
Quantity of basal food intake upon the utilization of vitamin A, effect of 13
Quotient, respiratory, and gaseous exchange in man, effect of various amounts of caffeine on 307

RABBIT, uncomplicated vitamin E deficiency in, and its relation to the toxicity of cod liver oil 225
Rat, rate of absorption of various fatty acids by 373
Rats, bones in, at conclusion of pregnancy, relation of parathyroid function and diet to mineral composition of 235
Rats, growing, severe calcium deficiency in. I. Symptoms and pathology 61
Rats, growing, severe calcium deficiency in. II. Changes in chemical composition 75
Rats, magnesium deficient, fasting catabolism and food utilization of 363
Rats, pregnant, effects of parathyroid deficiency and calcium and phosphorus of the diet on 179
Rats, rickets in, produced by high-calcium-low-phosphorus diets, effect of A.T.10 (dihydrotachysterol) on 147
Rats, young, hemorrhagic degeneration in, effect of supplementary choline, methionine and cystine and of casein, lactalbumin, fibrin, edestin and gelatin in 291
Rats, young, some dietary factors affecting incidence and severity of hemorrhagic degeneration in 633
Recovery, partial, without food, depletion of tissue glycogen during fasting and fatigue and 477
REIFMAN, AARON. See Deuel, Harry J., Jr. 373
Relation of parathyroid function and diet to the mineral composition of the bones in rats at the conclusion of pregnancy 235
Reproductive performance at restricted levels of carotene intake, carotene and vitamin A in cattle blood plasma with observations on 135
Requirement, calcium, of adult man and the utilization of the calcium in milk, further experiments on 577
Requirement, calcium, of man: Balance studies on seven adults 565
Requirement of mouse for pantothenic acid and for a new factor of the vitamin B complex 609
Requirements, nutritional, during the latter half of life 45
Respiratory metabolism in fructosuria 553
Respiratory quotient, and gaseous exchange in man, effect of various amounts of caffeine on 307
Response, biologic, of chickens to certain organic acids and salts with particular reference to their effect on ossification 515
Riboflavin and thiamin in the rumen content of cattle, studies on ... 85
Riboflavin as a factor in economy of food utilization 453
Riboflavin contents of some typical fruits 175
RICHARDSON, LUTHER R. See Hogan, Albert G. 327
Rickets, and cereals. XIII. Phytic acid, yeast nucleic acid, soybean phosphatides and inorganic salts as sources of phosphorus for bone calcification 213
Rickets in rats produced by high-calcium-low-phosphorus diets, effect of A.T.10 (dihydrotachysterol) on 147
RITCHIE, WALTER S. See Moore, Edwin L. 125
RITZMAN, ERNEST G. See Lee, Robert C. 321
ROSE, MARY SWARTZ 209
Rumen content of cattle, studies on riboflavin and thiamin in 85
Rumen content of sheep and cows fed vitamin-low diets, quantitative study of vitamins in. III. Thiamin 163
Rumen content of sheep and cows fed vitamin-low diets, quantitative study of vitamins in. IV. Pantothenic acid 405
RUTHERFORD, ESTHER. See Outhouse, Julia 565
RYNBERGEN, HENDERIKA J., WILLIAM H. CHAMBERS AND NORMAN R. BLATHERWICK. Respiratory metabolism in fructosuria 553

SALTS and organic acids, certain, with particular reference to their effect on ossification, biologic response of chickens to 515
Salts, inorganic, phytic acid, yeast nucleic acid and soybean phosphatides as sources of phosphorus for bone calcification 213
Salts, sodium and potassium, in the treatment of experimental diabetes and diabetes mellitus 599
SANDIA, JOSEPH G., AND LEOPOLD R. CERECEDO. Requirement of the mouse for pantothenic acid and for a new factor of the vitamin B complex 609
SCHALK, A. F. See Hunt, Charles H. 55
School children, group of, study of vitamin C nutrition in. Part II. Dietary evaluation 527
Severe calcium deficiency in growing rats. I. Symptoms and pathology 61
Severe calcium deficiency in growing rats. II. Changes in chemical composition 75
Severity, and incidence, of hemorrhagic degeneration in young rats, some dietary factors affecting ... 633
SHARPLESS, GEORGE R., AND MARGARET METZGER. Arsenic and goiter 341
Sheep and cows fed vitamin-low diets, quantitative study of vitamins in the rumen content of. III. Thiamin 163
Sheep and cows fed vitamin-low diets, quantitative study of vitamins in the rumen content of. IV. Pantothenic acid 405
Sheep, pig and goat, skin temperatures of, under winter conditions 321
SHERMAN, H. C. Mary Swartz Rose . 209
SHERMAN, HENRY C. See Briwa, Kathryn E. 155
SHERMAN, HENRY C. See Lanford, Caroline S. 175
SHERMAN, HENRY C. See Van Duyne, Frances O. 221
SHIELDS, JOHN B., AND HAROLD H. MITCHELL. The effect of calcium and phosphorus on the metabolism of lead 541
SHOHL, ALFRED T., AND SIDNEY FARBER. Effect of A.T.10 (dihydrotachysterol) on rickets in rats produced by high-calcium-low-phosphorus diets 147
Skin temperatures of the pig, goat and sheep under winter conditions ... 321
SNELL, ESMOND E., DONNA E. ALINE, JAMES R. COUCH AND PAUL B. PEARSON. The effect of diet on the pantothenic acid content of eggs 201
SODERSTROM, GUSTAVE F. See Hardy, James D. 383
Sodium and potassium salts in the treatment of experimental diabetes and diabetes mellitus 599
Some observations of vitamin B_6 deficiency in the dog 275
Soybean phosphatides, phytic acid, yeast nucleic acid and inorganic salts as sources of phosphorus for bone calcification 213
Sparing action of thiamine on body tissue catabolism 445
Specific dynamic effects of amino acids and their bearing on the causes of specific dynamic effects of proteins 257
SPERLING, GLADYS. See McCay, Clive M. 45
STEENBOCK, HARRY. See Krieger, Carl H. 213
STEGGERDA, FREDERIC R., AND HAROLD H. MITCHELL. Further experiments on the calcium requirement of adult man and the utilization of the calcium in milk 577
STEVEN, DAVID, AND GEORGE WALD. Vitamin A deficiency: A field study in Newfoundland and Labrador .. 461
STREET, HAROLD R., GEORGE R. COWGILL AND HARRY M. ZIMMERMAN. Some observations of vitamin B_6 deficiency in the dog 275
STROUSE, SOLOMON, FLORENCE BUEL, RAYMOND KAY AND DOUGLAS DRURY. Sodium and potassium salts in the treatment of experimental diabetes and diabetes mellitus 599
Studies, balance, on seven adults. Calcium requirement of man ... 565
Studies on riboflavin and thiamin in the rumen content of cattle ... 85
Studies on vitamin C metabolism of four preschool children 503
Study, field, in Newfoundland and Labrador. Vitamin A deficiency 461
Study of need for cobalt in dogs on milk diets 93
Study of vitamin C nutrition in a group of school children. Part II. Dietary evaluation 527
Study, quantitative, of vitamins in the rumen content of sheep and cows fed vitamin-low diets. III. Thiamin 163
Study, quantitative, of vitamins in the rumen content of sheep and cows fed vitamin-low diets. IV. Pantothenic acid 405
SURE, BARNETT, AND MAURICE DICHEK. The sparing action of thiamine on body tissue catabolism 445
SURE, BARNETT, AND MAURICE DICHEK, with the assistance of Martin M. Citron. Riboflavin as a factor in economy of food utilization 453
Swine, optimum level of protein intake for the growth and fattening of .. 419
Symptoms and pathology. Severe calcium deficiency in growing rats. I. 61

TEETH, mature, on the manner of acquisition of fluorine by 35
Temperatures from 22°C. to 35°C., basal metabolism and heat loss of young women at 383
Temperatures, skin, of the pig, goat and sheep under winter conditions 321
Thiamin. A quantitative study of vitamins in the rumen content of sheep and cows fed vitamin-low diets. III. 163
Thiamin, and riboflavin, in the rumen content of cattle, studies on . 85
Thiamine, sparing action of, on body body tissue catabolism 445
Thiamine, urinary excretion of, on high fat and high carbohydrate diets 411
THOMPSON, C. RAY. See Krieger, Carl H. 213
Tissue catabolism, body, sparing action of thiamine on 445

Tissue glycogen, depletion of, during fasting and fatigue and partial recovery without food 477
TOEPFER, EDWARD W. See Van Duyne, Frances O. 221
Toxicity of cod liver oil, uncomplicated vitamin E deficiency in the rabbit and its relation to 225
Treatment of experimental diabetes and diabetes mellitus, sodium and potassium salts in 599

UNCOMPLICATED vitamin E deficiency in the rabbit and its relation to the toxicity of cod liver oil 225
Urinary excretion of thiamine on high fat and high carbohydrate diets 411
Urine and blood constituents, and heat production, after administration of l(—)histidine to the dog 25
Utilization, food, and fasting catabolism, of magnesium deficient rats ... 363
Utilization, food, riboflavin as a factor in economy of 453
Utilization of calcium in milk, further experiments on the calcium requirement of adult man and 577
Utilization of calcium of milk by adults 351
Utilization of vitamin A, effect of quantity of basal food intake upon 13

VAN DUYNE, FRANCES O., CAROLINE S. LANFORD, EDWARD W. TOEPFER AND HENRY C. SHERMAN. Lifetime experiments upon the problem of optimal calcium intake 221
Vitamin A, and carotene, in cattle blood plasma with observations on reproductive performance at restricted levels of carotene intake 135
Vitamin A deficiency: A field study in Newfoundland and Labrador 461
Vitamin A, effect of quantity of basal food intake upon utilization of 13
Vitamin A in liver, effect of certain carcinogens on 431
Vitamin B complex, new factor of, requirement of mouse for pantothenic acid and for 609
Vitamin B_6 deficiency in dog, some observations of 275
Vitamin B_6 (pyridoxine), distribution of, in meat and meat products ... 589
Vitamin C metabolism of four preschool children, studies on 503
Vitamin C nutrition in a group of school children, study of. Part II. Dietary evaluation 527
Vitamin E deficiency, uncomplicated, in the rabbit and its relation to the toxicity of cod liver oil ... 225
Vitamin K bioassay of 1
Vitamin deficiency, perosis due to ... 327
Vitamin-low diets, quantitative study of vitamins in the rumen content of sheep and cows fed. III. Thiamin 163
Vitamin-low diets, quantitative study of vitamins in the rumen content of sheep and cows fed IV. Pantothenic acid 405

WAISMAN, HARRY A. See Henderson, LaVell M. 589
WALD, GEORGE. See Steven, David .. 461
Wernicke's disease of man, B_1 avitaminosis analogous to. A deficiency disease of foxes produced by feeding fish 243
Winter conditions, skin temperatures of the pig, goat and sheep under 321
Women, young, basal metabolism and heat loss of, at temperatures from 22°C. to 35°C. Clinical calorimetry no. 54 383
WYNN, WINFREY. See Haldi, John .. 307

YEAST nucleic acid, phytic acid, soybean phosphatides and inorganic salts as sources of phosphorus for bone calcification ... 213
Young rats, hemorrhagic degeneration in, effect of supplementary choline, methionine and cystine and of casein, lactalbumin, fibrin, edestin and gelatin in 291
Young rats, some dietary factors affecting incidence and severity of hemorrhagic degeneration in 633
Young women, basal metabolism and heat loss of, at temperatures from 22°C. to 35°C. Clinical calorimetry no. 54 383

ZIMMERMAN, HARRY M. See Street, Harold R. 275